ACTA PHYSIOLOGICA SCANDINAVICA

ACTA PHYSIOL. SCAND

VOL 76

Vol. 76. INDEX

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From the Neurosurgical Clinic A, University Hospital of Lund, and Institute of Anatomy and Histology, University of Lund, Sweden

Possible Relation between the Degree of Cardiac Adrenergic Innervation and the Resistance to Hypothermic Ventricular Fibrillation in Young Cats

By

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Received 27 June 1968

Abstract

NIELSEN, KAI C *Possible relation between the degree of cardiac adrenergic innervation and the resistance to hypothermic ventricular fibrillation in young cats* Acta physiol scand 1969 76 1—9

nerves compared with adult animals, in agreement with a lower concentration of cardiac noradrenaline The possibility is discussed that the not yet fully developed adrenergic innervation of the heart in young animals may account for the higher resistance to reduction of the body temperature as compared with adult animals

A recent series of investigations on cats subjected to hypothermia has shown that interference with the adrenergic nerves through depletion of their transmitter stores (Nielsen and Owman 1965, 1967b, 1968b) or blockade of their transmitter release (Nielsen and Owman 1968a) effectively controls the spontaneous ventricular fibrillation invariably occurring in untreated control animals at a body temperature of about 21° C Since also blockade of the adrenergic β receptors (Nielsen and Owman 1965, 1966) and surgical cardiac sympathectomy (Nielsen and Owman 1968c) are effective in preventing the ventricular fibrillation at reduced body temperature, there is strong reason to believe that the adrenergic nerves in the *heart* are of considerable importance for the development of this major complication of induced hypothermia This has been further confirmed in *in vitro* experiments on isolated heart preparations (Falck *et al* 1968)

It has long been known that young animals survive cooling to low body temperature much better than adult animals (see Prokopeva 1960). In view of the fact that blockade of the adrenergic influences to the heart muscle in cats prevents the development of hypothermic ventricular fibrillation as discussed above and with regard to the finding that hibernators, in contrast to non-hibernators, have a remarkably scarce *adrenergic innervation in the myocardium* (Nielsen and Owman 1965, 1968d) it would seem of interest to elucidate whether the resistance of young animals to systemic cooling could be correlated with a not yet fully developed adrenergic innervation of the heart.

Material and methods

12 kittens of either sex and weighing 0.5—1.0 kg (corresponding to an age of about 5 to 10 weeks) were anesthetized with nembutal (30 mg/kg intraperitoneally) and cooled by submersion in an ice water bath of 3—9° C. Respiration, rectal temperature, arterial blood pressure and ECG (standard limb lead II) were continuously recorded (for details see Nielsen and Owman 1967b). When blood pressure fell below a pre-determined level of 60 mm Hg accompanying the progressive fall in body temperature, metaraminol (Aramine, Merck Sharp & Dohme) was given as an *intermittent i.v. infusion* (10 μg/ml of 10 % invertose; see Nielsen and Owman 1967b) in order to maintain a sufficient blood supply to *e.g.* the brain (Haggendal 1965). Artificial respiration was applied with a pump respirator connected to an endotracheal T-tube when spontaneous breathing became inadequate. Active cooling was interrupted at 18° C rectal temperature and the ice water was replaced with warm water of 34—42° C. Rectal temperature continued to fall to 17.4—16.2° C before it reversed; the animals were then re-warmed to normothermia.

After reaching normothermia, the animals were bled to death and the heart was removed. *Pieces from the atria and ventricles were quenched to the temperature of liquid nitrogen*, freeze-dried, treated with formaldehyde gas for the histochemical demonstration of the adrenergic transmitter (Falck 1962, Falck *et al.* 1962, Corrodi and Hillarp 1963, 1964) and further processed for fluorescence microscopic analysis (for details see Falck and Owman 1965).

The number and intensity of the fluorescent cardiac adrenergic nerves in the cooled kittens were compared with the nerves in pieces of heart obtained from 4 untreated kittens (weighing between 0.5 and 0.8 kg) and 4 adult cats of 2.7—3.8 kg. The remaining heart tissue from these control animals was used for chemical estimation of the noradrenaline concentration according to Bertler *et al.* (1958) and Häggendal (1963). These animals too were anesthetized with nembutal.

Results

Hypothermia. Of the 12 animals subjected to hypothermia, only one (no. 5, Table I) developed ventricular fibrillation (at a rectal temperature of *18° C, Fig. 1*). The remaining animals reached rectal temperatures of 17.4 to 16.2° C (Table I) without major cardiac complications other than rhythmic irregularities. All except 2 animals (no. 2 and 10) had to receive metaraminol as an intermittent infusion to maintain the blood pressure above 60 mm Hg (Table I). Characteristically, however, the animals did not respond to metaraminol administration at rectal temperatures below 22—19° C during cooling (Fig. 1). When the body temperature reversed during re-warming, the blood pressure spontaneously picked up rapidly and the animals responded with a marked increase in blood pressure if metaraminol infusion was necessary, even at the low rectal temperatures. In one of the animals (no. 3) the blood pressure could not be maintained at an adequate level during re-warming and the

TABLE I Synopsis of the 12 kittens subjected to hypothermia. Lowest rectal temperature (before it reversed, except in animal no. 5), total amount of metaraminol administered, number of fluorescent nerves estimated in entire heart, fluorescence intensity of cardiac adrenergic nerves.

Kitten No.	Weight (kg)	Lowest rectal temp. (°C)	Amount of metaraminol (μg)	Total number of fluorescent nerves[b]	Fluorescence intensity
1	0.9	16.2	100	++	reduced
2	1.0	17.1	0	+	reduced
3	0.9	17.0	200	+	reduced
4	0.7	16.8	10	++	not reduced
5	0.7	18.0[a]	50	++	reduced
6	0.7	16.2	125	+	reduced
7	0.7	17.3	150	++	not reduced
8	0.6	16.8	40	++	not reduced
9	0.5	16.2	60	+	reduced
10	0.9	17.4	0	++	reduced
11	0.7	17.0	60	+	not reduced
12	0.7	17.0	50	+++	reduced

[a] This was the only animal that developed ventricular fibrillation.
[b] *Estimated on the basis of* ++++ *for adult animals.*

experiment was discontinued at 29° C rectal temperature. Autopsy revealed a profuse peritoneal bleeding from a rift in the liver, probably produced during the intraperitoneal administration of the anesthetic.

Cardiac adrenergic nerves. Under the conditions used, the adrenergic transmitter substance present throughout the sympathetic nerve endings exhibits an intense green fluorescence (Falck 1962). In conformity with previous observations on cats (Ehinger *et al.* 1966, Jacobowitz *et al.* 1967, Nielsen and Owman 1967b) the heart of the adult control animals had a fairly rich adrenergic innervation. The terminals partly enclosed the blood vessels, particularly the arteries and arterioles, with dense plexuses superimposed on the media, and were partly directed to the cardiac muscle fibres which they were seen to follow closely for long distances (Fig. 1a). The over-all density of adrenergic innervation was highest in the atria. The wall of the right ventricle contained a moderate number of terminals, whereas the innervation was less pronounced in the left ventricular wall.

Analysis of the heart preparations from the 12 kittens subjected to hypothermia (and the 4 control kittens) usually revealed a considerably smaller amount of adrenergic nerve terminals (Fig. 2b). Thus, only very few fluorescent fibres were present in all parts of the heart in animals no. 2, 3, 6 (Fig. 2c), 9 (Fig. 2d) and 11 (see Table I). Most of the nerves were clearly related to the blood vessels, whereas few, or almost no fluorescent nerves were found in the myocardium proper (Fig. 1c

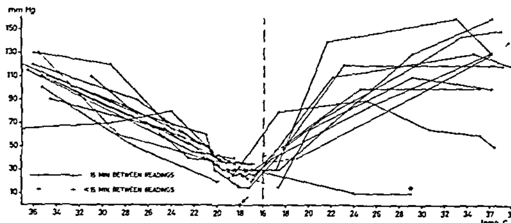


Fig 1 Blood pressure rectal temperature and cooling time of the 12 kittens subjected to hypothermia. Only one animal developed ventricular fibrillation at 18° C rectal temperature (⟶) The blood pressure could not be maintained in one animal during re warming owing to severe peritoneal bleeding the experiment was discontinued at 29° C (*)

and d) The number of nerves was somewhat larger in 6 of the animals no 1, 4 (Fig 3a) 5 7 8 and 10 (Fig 3b) This was especially evident in the atrial portions The innervation was also in these animals much less developed among the cardiac muscle fibres In only one of the animals subjected to hypothermia (no 12, Fig 3c) did the number and distribution of the cardiac adrenergic nerves resemble that of the adult animals

The low degree of development of the adrenergic innervation in the heart was evident in all the control kittens that were not subjected to hypothermia (Fig 2b) The nerves had a similar varicosed appearance characterizing the terminals in adult individuals although in the preparations from the kittens the varicosities seemed smaller they had a less bright fluorescence and were more irregularly interspersed along the nerve terminal It was clearly evident that in most of the animals in the *hypothermia group the fluorescence intensity of the nerves was slightly to markedly reduced in comparison with the untreated controls* In animals no 4 7, 8 and 11, however did the intensity of the emitted light appear to be unaffected (Table I)

In agreement with the smaller number of cardiac adrenergic nerves in young compared with adult animals fluorimetric determinations showed a distinct difference in the amount of noradrenaline (mean ± standard error of the mean of μg noradrenaline per g tissue) young atria 0 87 ± 0 07 and ventricles 0 70 ± 0 10 to be compared with adult atria 1 40 ± 0 11 and ventricles 1 72 ± 0 08

As in the adult animals no fluorescent nerve cells could be recognized in any part of the heart. However large formations of non fluorescent probably cholinergic ganglion cells occurred in the right atrial wall near the caval inflow Scattered among these cells were found groups of small intensely green fluorescent cells some of

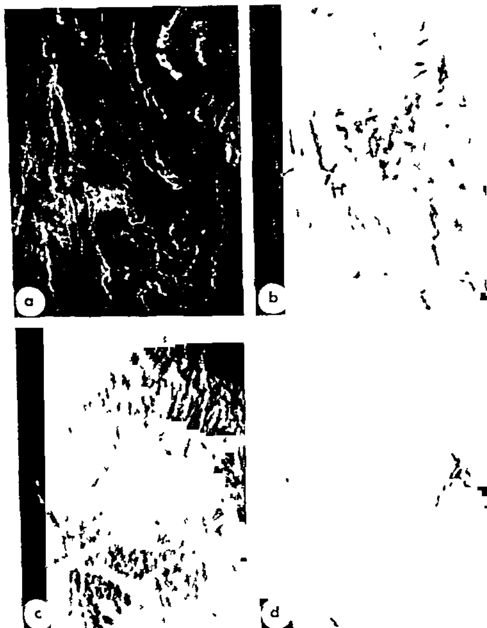


Fig 2 Fluorescence photomicrographs of preparations from right ventricle 150× (a) Untreated adult animal fairly large number of terminals to vessels and musculature (b) Untreated kitten fewer nerve terminals usually related to vessels (c) Kitten no 6 and (d) kitten no 9 very few nerves only at vessels

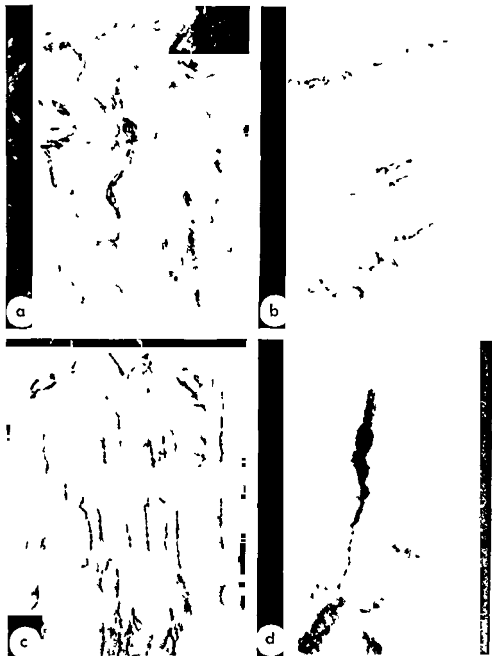


Fig 3 Fluorescence photomicrographs of preparations from right ventricle 150× (a) Kitten no 4 and (b) kitten no 10 the innervation is better developed than in kittens no 6 and 9 most nerves still related to vessels (c) Kitten no 12 fairly well developed innervation (d) Kitten no 9 Two intensely greenfluorescent small cells in right atrium One of the cells issues a long beaded process Cell nucleus not visible owing to the high fluorescence intensity 300×

which were seen to issue a process of considerable length, this sometimes even seemed to have a varicosed appearance (Fig 3d)

Large bundles of preterminal sympathetic nerves, having a characteristically smooth appearance and low fluorescence intensity, coursed along large arteries particularly in the atria, but also in the walls of the ventricles near the intraventricular septum

Discussion

Only one out of the 12 kittens subjected to hypothermia down to 17 4—16 2° rectal temperature developed spontaneous ventricular fibrillation, whereas the remaining animals could be re warmed to normothermia without this cardiac complication This contrasts sharply with the constant development of ventricular fibrillation seen in several series of experiments on adult cats similarly cooled without previous treatment (Nielsen and Owman 1966, 1967b, 1968a—c) On the other hand, blockade of the sympatho-adrenal influences on the cardiac β receptors offers an efficient protection against the spontaneous hypothermic ventricular fibrillation (Varma *et al* 1963, Nielsen and Owman 1965, 1966, 1967b, 1968a—c, Bennett *et al* 1968 Falck *et al* 1968) In view of these findings it seems highly significant that hibernators such as the bat, hedgehog, and thirteen lined ground squirrel have only a slight—if any—adrenergic innervation to the ventricular muscle cells (Nielsen and Owman 1965, 1968d) in contrast to the well developed adrenergic innervation in the myocardium of non hibernating mammals (Angelakos *et al* 1963, Dahlström *et al* 1965, Ehinger *et al* 1966, Jacobowitz *et al* 1967, Nielsen and Owman 1967b) These results make it highly probably that the present histochemical findings of an incomplete cardiac adrenergic innervation, particularly to the muscle fibres of young cats offer one reasonable explanation for the resistance of such animals to the development of ventricular fibrillation during deep hypothermia However, it should be borne in mind that also other circumstances may contribute to this marked difference between young and adult animals, for example, differences in the experimental conditions such as a shorter cooling time of the smaller young animals or developmental differences in the degree of myocardial hyperexcitability during cooling another important factor in the genesis of hypothermic ventricular fibrillation (Brooks 1956 Hegnauer and Covino 1956, Hegnauer and Angelakos 1959 Hoffman 1959)

The adrenergic nerves present in the heart tissues of animals subjected to hypothermia often had a lower transmitter content as revealed by the reduced fluorescence intensity, compared with the corresponding untreated control animals Part of this noradrenaline release may be a consequence of the metaraminol administration (*cf* Andén 1964) but a reduced noradrenaline fluorescence was evident also in those two animals that received no metaraminol during the experiment This confirms previous findings on adult cats that have been subjected to the same degree of hypothermia and shows at a neuronal level that cooling enhances the output of noradrenaline from the adrenergic nerves (Goldstein and Nakajima 1966 Nielsen and Owman 1967b However it can be supposed that the noradrenaline concentra-

tion to which the myocardial receptors are exposed is too low to precipitate ventricular fibrillation in the *young* animals This is analogous to the findings that differential depletion of catecholamines from the well-developed system of cardiac sympathetic nerves in *adult* animals (Nielsen and Owman 1967a) effectively prevents the appearance of ventricular fibrillation during hypothermia (Nielsen and Owman 1965, 1967b).

From a developmental point of view it should be noted that the establishment of the adrenergic innervation apparatus of the heart appears to be a comparatively late phenomenon. This agrees with findings on the chicken embryo in which the cardiac adrenergic nerve supply has been found to lag behind the development of sympathetic nerve terminals in certain other structures (Enemar *et al* 1965) The present histochemical data indicate that the adrenergic nerve plexuses are at first formed around the cardiac vessels, from which nerve terminals then reach into the myocardium proper This pattern of development seems to proceed in direction from the atria to the ventricles

Supported by grants from the Association for the Aid of Crippled Children New York, Ollie and Elof Ericsson s Foundation, and Anna Lisa and Sven Eric Lundgren s Foundation

References

ANDÉN N E On the mechanism of noradrenaline depletion by *alpha* methyl metatyrosine and metaraminol. *Acta pharmacol* (Kbh.) 1964 *21* 260—271

ANGELAKOS E T K FUXE and M L TORCHIANA Chemical and histochemical evaluation of the distribution of catecholamines in the rabbit and guinea pig hearts *Acta physiol scand* 1963 *59* 184—192

BENNETT C R. H H SMOOKLER and J P BUCKLEY Prevention of ventricular fibrillation during induced hypothermia by N,N-diisopropyl N isoamyl N -diethylaminoethylurea (P 286) *J Pharmacol exp Ther* 1968 *159* 115—122

BERTLER Å A. CARLSSON E ROSENGREN and B WALDECK A method for the fluorimetric determination of adrenaline noradrenaline and dopamine in tissues *Kungl Fysiogr Sällsk Lund Förh* 1958 *28* 121—123

BROOKS C M Hypothermia and the physiology of cardiac excitability *In The Physiology of Induced Hypothermia* Ed. by Dripps R. D Nat Acad Sci Nat Res. Counc Washington, 1956 287—301

CORRODI, H and N Å HILLARP Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen 1 Identifizierung der fluoreszierenden Produkte aus Modellversuchen mit 6 7 Dimethoxyisochinolinderivaten und Formaldehyd *Helv chim Acta* 1963 *46* 2425—2430

CORRODI, H and N Å HILLARP Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen 2 Identifizierung des fluoreszierenden Produktes aus Dopamin und Formaldehyd. *Helv chim Acta* 1964 *47* 911—918

DAHLSTRÖM A K FUXE M MYA TU and B E M ZETTERSTRÖM Observations on the adrenergic innervation of the dog heart *Amer J Physiol* 1965 *209* 689—692

EHINGER B B FALCK and B SPORRONG Adrenergic fibres to the heart and to peripheral vessels. *Bibl anat* 1966 *8* 35—45

ENEMAR, A., B FALCK and R HÅKANSON Observations on the appearance of norepinephrine in the sympathetic nervous system of the chick embryo *Develop Biol* 1965 *11* 268—283

FALCK, B Observations on the possibilities of the cellular localization of monoamines by a fluorescence method *Acta physiol scand* 1962 *56* Suppl 197 1—25

FALCK B, N Å. HILLARP G THIEME and A TORP Fluorescence of catecholamines and related compounds condensed with formaldehyde *J Histochem Cytochem* 1962 *10* 348—354

FALCK, B, K. C NIELSEN CH OWMAN H PERSSON and B SPORRONG In preparation 1968

Fa ... A detailed methodological description of the fluorescence method
—23
G ... echol

Häggendal, E, Blood flow autoregulation of the cerebral grey matter with comments on its mechanism *Acta neurol scand* 1965 Suppl 14 104—110
Häggendal, J, An improved method for fluorimetric determination of small amounts of ...

...

Hoffman, B F, Hypothermia and vulnerability *Ann N Y Acad Sci* 1959 *80* 348—350
Jacobowitz, D, Th Cooper and H B Barner, Histochemical and chemical studies of the localization of adrenergic and cholinergic nerves in normal and denervated cat hearts *Circ Res* 1967 *20* 289—298
Nielsen, K C and Ch Owman, On the control of ventricular fibrillation during hypothermia *Communic Dept Anat Univ Lund* 1965 *6* 1—13
Nielsen, K C and Ch Owman, Control of ventricular fibrillation by a *beta* adrenergic blocking agent (INPEA) during induced hypothermia in cats *Life Sci* 1966 *5* 1611—1623
Nielsen, K C and Ch Owman, Differential amine depletion from cardiac adrenergic nerves by Segontin *Experientia* (Basel) 1967a *23* 203—204
Nielsen, K C and Ch Owman, Control of ventricular fibrillation during induced hypothermia in cats after differential depletion of cardiac catecholamine stores with prenylamine (Segontin) *Circulat Res* 1967b *21* 45—58
Nielsen, K C and Ch Owman, Control of ventricular fibrillation during induced hypothermia in cats after blocking the adrenergic neurons with bretylium *Life Sci* 1968a *7* 159—168
Nielsen K C and Ch Owman, Effect of reserpine on the spontaneous ventricular fibrillation developing during induced deep hypothermia in cats *Arch int Pharmacodyn* 1968b *175* 412—421
Nielsen K C and Ch Owman, Control of spontaneous ventricular fibrillation during induced hypothermia in cats by acute cardiac sympathectomy *Acta physiol scand* 1969 *76* 73—81
Nielsen, K C and Ch Owman, Difference in cardiac adrenergic innervation between hibernators and non hibernating mammals *Acta physiol scand* 1968d Suppl 316 1—30
Prokopeva E M, The limits of overcooling and survival in puppies *In The Problem of* ... Oxford 1960 214—236
... ationship of the catecholamines ... ermia in dogs *Canad J Bio chem Physiol* 1963 41 301—307

Acta physiol scand. 1969. 76. 10—23

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Renal Autoregulation during Infusion of Noradrenaline, Angiotensin and Acetylcholine

By

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Received 6 July 1968

Abstract

KIIL F J KJEKSHUS and E LØYNING *Renal autoregulation during infusion of noradrenaline, angiotensin and acetylcholine* Acta physiol scand 1969 76 10—23

To examine whether renal autoregulation was abolished by vasoconstriction and vasodilatation experiments were performed on anesthetized dogs with intact renal circulation and nervous supply Angiotensin or noradrenaline was infused into the renal artery at a constant rate that reduced renal blood flow (RBF) to less than half of control values By mechanical constriction of the renal artery perfusion pressure could be reduced to averages of 69 and 80 mm Hg respectively (74 mm Hg during control conditions) before RBF dropped more than 5 % Normal autoregulation was also obtained during intravenous infusion of angiotensin and noradrenaline during stimulation of renal nerves and after development of tachyphylaxis to angiotensin At normal and low arterial pressures but at similar RBF noradrenaline or angiotensin was injected into the renal artery RBF was least reduced at low perfusion pressure indicating that autoregulation did not compensate for the vasoconstrictive effect of noradrenaline and angiotensin By acetylcholine infusion RBF could be doubled and a curvilinear relationship between RBF and perfusion pressure was obtained Similar curves could be constructed by the application of Poiseuille's law on the premise that vasoactive agents act mainly on muscle elements other than those participating in autoregulation It is concluded that autoregulation is essentially independent of nervous and humoral stimuli

Renal blood flow (RBF) and glomerular filtration rate (GFR) remain constant when renal perfusion pressure is lowered to 70—80 mm Hg by mechanical constriction of the renal artery or aorta above the renal artery Autoregulating mechanisms which dilate the afferent arterioles are then maximally activated and the resistance in the preglomerular vessel probably remains unchanged when perfusion pressure is further reduced Whether nervous and humoral stimuli interfere with renal autoregulation in the intact organism has not yet been established and it is accordingly undecided whether renal autoregulation is of any significance in circulatory homeostasis

Previous studies of the effects of vasoactive agents on renal autoregulation have

been performed mainly on completely isolated or artificially perfused kidneys that usually have low RBF at control pressure. Vasoconstrictors have been shown to improve (Schmid, Garrett and Spencer 1964) and abolish renal autoregulation (Folkow and Langston 1964), whereas acetylcholine and other vasodilators have been uniformly found to abolish autoregulation (Waugh 1958, Thurau and Kramer 1959, Nahmod and Lanari 1964).

In the present study, renal perfusion pressure was mechanically lowered in dogs with intact circulation during infusion of angiotensin, noradrenaline or acetylcholine into the renal artery, or during intravenous infusion of angiotensin or noradrenaline. Renal autoregulation was also examined during stimulation of renal nerves. In order to examine a proposal of Green, Rapela and Conrad (1963) that the autoregulating mechanism reduces the effects of vasoconstrictive agents by providing a counter dilatation, noradrenaline or angiotensin was injected into the renal artery in similar doses at normal and low perfusion pressures.

Methods

Mongrel dogs weighing 15 to 30 kg were anesthetized with intravenous sodium pentobarbital at a dosage of 30 mg/kg b.w. and maintained by additional doses of 1 to 2 mg/kg b.w. during the experiment which usually lasted over 3 hrs. The left kidney was exposed retroperitoneally through a flank incision and the renal artery cleared of surrounding tissue by blunt dissection for a length of 5—6 cm. Small nerves running along the artery were left intact. The fat pad surrounding the kidney was left undisturbed and the ureter was not cannulated. Renal arterial pressure was obtained through a 20 gauge needle introduced as close as possible to the kidney and connected to a polyethylene catheter, and aortic pressure through a polyethylene catheter inserted below the renal arteries. Mean arterial pressures were measured with Statham transducers and recorded continuously on a Sanborn recorder. The pressure difference between aorta and the distal part of the renal artery was 10 to 15 mm Hg at control flows. A polyvinyl tubing with outer diameter 0.4 mm was introduced into the renal artery *ad modum* Herd and Barger (1964) with the tip directed upstream, and angiotensin (Hypertensin CIBA), noradrenaline or acetylcholine infused into the renal artery in isotonic saline at rates up to 6 ml/min by means of an electric constant delivery pump.

Renal perfusion pressure was reduced in most experiments by a plastic adjustable clamp placed immediately distal to the tip of the tubing used for infusions. In 2 dogs, renal perfusion pressure was reduced by constricting aorta above the origin of the renal arteries with a Blalock clamp. The effect on autoregulation of infusing noradrenaline or angiotensin at a constant rate into vena cava was examined in 4 dogs. In these experiments, renal perfusion pressure was reduced by inflating a balloon introduced from the femoral artery into the aorta above the renal arteries.

RBF was measured with a square wave electromagnetic flowmeter (Nycotron, Oslo) and recorded on a Sanborn recorder. A flowmeter probe, selected on the basis of vessel size (gap diameter 3—4.5 mm), was placed on the renal artery as close as possible to the aorta. Zero flow reading was checked by occluding the renal artery. Readjustment was rarely necessary. The flow probes were calibrated directly on femoral or carotid arteries of similar size in other experiments.

In 2 dogs with two main renal arterial branches, the adjustable clamp was placed on the common renal arterial stem and pressure recorded distal to the clamp. Arterial flow was recorded from both branches. A catheter for infusion of vasoactive agents was inserted into only one of the branches.

Following surgical procedures, 10—20 min were allowed before control autoregulation curves were obtained. Constriction of the renal artery or aorta was performed with the kidney in its normal position and with the incision partially closed by means of wound clamps. Renal perfusion pressure was either lowered continuously over several minutes or in steps of 5—10 mm Hg.

At the end of three experiments, the bundles of nerves accompanying the renal vessels were dissected free, sectioned, and the distal end hooked to electrodes and stimulated (6 V, 10 cps) with a Grass stimulator while renal autoregulation was examined.

TABLE I Renal blood flow at control and lowest autoregulating perfusion pressures before and during intra arterial infusion of angiotensin or noradrenaline

		No infusion		Angiotensin		Noradrenaline	
		Control	Constriction	Control	Constriction	Control	Constriction
Dog 1							
14 5 kg	RPP	150	85	165	75	160	85
	RBF	170	160	85	85	80	75
Dog 2							
27 kg	RPP	120	65	130	60	125	70
	RBF	250	245	90	85	120	120
Dog 3							
19 kg	RPP	115	70	130	70	130	80
	RBF	155	150	60	60	75	75
Dog 4							
26 kg	RPP	165	80	165	75	170	90
	RBF	3 0	340	215	210	225	205
Dog 5							
22 kg	RPP	120	70			120	75
	RBF	470	455			360	350
Dog 6							
18 kg	RPP	165	85			165	95
	RBF	230	215			135	130
Dog 7							
28 kg	RPP	125	65	125	65		
	RBF*	185	185	80	75		
	RPP	130	75			130	75
	RBF*	150	155			90	80
Dog 8							
20 kg	RPP	150	75	150	70	150	75
	RFB*	90	90	50	45	70	70

RPP Renal perfusion pressure mm Hg RBF Renal arterial blood flow (ml/min
RBF* Blood flow (ml min in a branch of the renal artery

Results

Control studies In control periods in 8 dogs RBF ranged between 155 and 470 ml/min (9 3—21 4 ml/kg b w Renal vascular resistance decreased during constriction of the renal artery, but the efficiency of autoregulation varied between animals The lowest autoregulating pressure averaged 74 mm Hg varying in the course of the experiments by 5—15 mm Hg which was unrelated to slight spontaneous changes

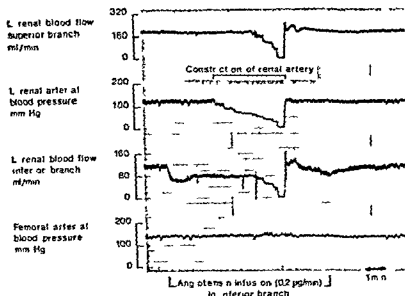


Fig 1 Blood flow in two arterial branches during stepwise constriction of common renal artery stem Maintenance of autoregulation is demonstrated during angiotensin infusion into the inferior branch the superior branch serving as control.

in RBF Lowering renal perfusion pressure had no consistent effect on RBF in the subsequent control periods and the lowest autoregulating pressure was not significantly higher at the end of the experiment

Table I shows mean control perfusion pressures and the lowest autoregulating mean pressure with associated RBF values On average RBF decreased less than 4 % (from 230 to 222 ml/min) during reduction of renal arterial perfusion pressure from a mean value of 138 to 74 mm Hg

The lowest autoregulating perfusion pressure was usually determined by reducing the perfusion pressure at a rate of 20—30 mm Hg/min. Typical recordings are shown in the upper parts of Fig 1 and 4 obtained from studies on dogs with two arterial branches RBF remained essentially constant down to perfusion pressures of 65 and 75 mm Hg respectively and fell rapidly with further reduction of perfusion pressure Infusion of angiotensin or noradrenaline into one arterial branch did not affect RBF or autoregulation in the other branch

Angiotensin Renal autoregulation was examined in 4 dogs during intraarterial infusion of angiotensin which reduced RBF to 36—58 % of control values and in 2 dogs with two renal arterial branches when RBF was reduced to 43 and 55 % of control flow respectively Control pressures and lowest autoregulating pressures are shown in Table I Infusion of angiotensin at a rate of 0 2 to 0 6 μg/min increased arterial perfusion pressure by an average of 6 mm Hg Renal autoregulation was present in all studies and RBF was maintained on average to a perfusion pressure of

TABLE II Renal blood flow at control and lowest autoregulating perfusion pressure Mean values during control conditions and during intra arterial and intravenous infusions of angiotensin and noradrenaline

		Control		Angiotensin		Noradrenaline	
		Control	Constriction	Control	Constriction	Control	Constriction
Intraarterial infusion	RPP	138	74	144	69	144	80
	RBF	231	222	98	93	144	138
			Inflation of balloon		Inflation of balloon		Inflation of balloon
Intravenous infusion	RPP*	118	85	170	85	185	80
	RBF	190	183	100	80	153	133

RPP Renal perfusion pressure (mm Hg) RBF Renal blood flow (ml/min) RPP* Aortic pressures

69 mm Hg which in each dog was as low as, or lower than the lowest autoregulating pressure during control conditions The oscillatory pattern after release of constriction was not consistently different from that observed in control conditions Recordings from one of the experiments are shown in Fig 1

Similar results were obtained during intravenous infusion of angiotensin (1—3 μg/min) RBF was on average slightly more than 50 % of control values Mean arterial perfusion pressure increased to 170 mm Hg Renal autoregulation was present down to perfusion pressures as low as during control conditions Mean values of all studies are shown in Table II

Tachyphylaxis to angiotensin developed rapidly during intra arterial infusion of angiotensin, and often prevented a persistent reduction of RBF to less than half of control values Recordings from a typical experiment are shown in Fig 2 where trebling the rate of angiotensin administration did not cause a further reduction in RBF An even more dramatic loss of vascular sensitivity was demonstrated in other experiments since the effect on RBF of a tenfold increase in angiotensin administration was only transient. Renal autoregulation was unaffected by development of tachyphylaxis By further reduction of perfusion pressure the vasoconstrictive effect of angiotensin was less pronounced RBF was zero at a perfusion pressure of 18 mm Hg which was not significantly higher than the closing pressure in control conditions

Renal nerve stimulation In several experiments in each of 3 dogs RBF was reduced to less than 60 % of control values by faradic stimulation of the nerves of the renal pedicle Renal autoregulation was present in all studies without significant changes of the lowest autoregulating pressure Release of constriction was followed by typical flow overshoot. Recordings from one of the studies are shown in Fig 3

Noradrenaline In 6 dogs, renal autoregulation was examined during intra arterial

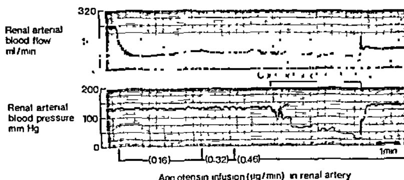


Fig 2 Intact pressure flow relationship during development of renal tachyphylaxis to angiotensin

infusion of noradrenaline which reduced RBF to 47—77 % of control values (Table I) In 2 dogs, infusion into one of the branches of the renal artery reduced RBF to 64 and 78 % of control values Intravenous infusion in 2 other dogs increased mean renal arterial pressure on average from 118 to 185 mm Hg, and reduced RBF to an average of 81 % of control flow (Table II) Renal autoregulation was present in all experiments During intravenous infusion, the lowest autoregulating perfusion pressures were as low as in control conditions, but were higher in 5 of 8 dogs during intra-arterial infusion, averaging 80 mm Hg, as against 74 mm Hg under control conditions (Table II)

All experiments showed that noradrenaline and angiotensin had different effects on RBF at or below the lowest autoregulating pressure Fig 4 shows recordings when noradrenaline was infused into one renal arterial branch while the other served as control By gradually constricting the main stem of the renal artery, RBF in both branches remained essentially unchanged to a perfusion pressure of 75 mm Hg The effect of a further slight reduction of renal perfusion pressure was different In the noradrenaline infused branch, RBF fell, and continued to fall, despite restoration of

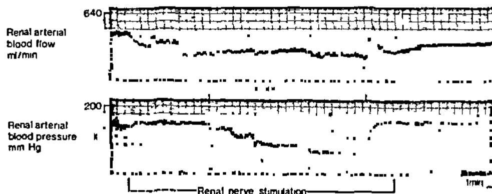


Fig 3 Presence of autoregulation during stimulation of distal end of renal nerves

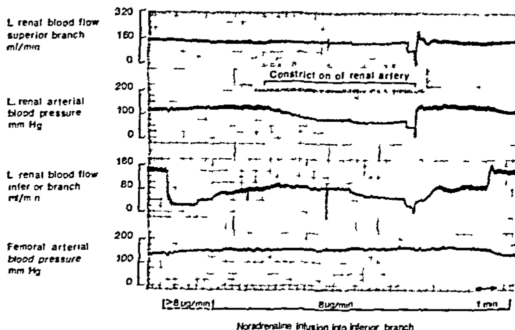


Fig 4 Blood flow in two arterial branches during stepwise constriction of common renal artery stem. Maintenance of autoregulation is demonstrated during noradrenaline infusion into the inferior branch, the superior branch serving as control

perfusion pressure to 75 mm Hg In 4 dogs RBF became zero when perfusion pressure was reduced to an average of 48 mm Hg (against 18 mm Hg during angiotensin infusion and under control conditions) After release of constriction several minutes *elapsed before control RBF was approached in noradrenaline-infused arteries* whereas RBF was rapidly restored during angiotensin infusion However if constriction was released at perfusion pressures within the autoregulating range the post release flow patterns were not significantly different during noradrenaline and angiotensin infusion

Acetylcholine By infusing acetylcholine into the renal artery at a rate of 2—10 μg/min RBF increased to a new stable level in less than 1 min In 4 dogs RBF could be increased to 140—350 % of control flow before systemic arterial mean pressure fell In all experiments RBF was markedly increased at perfusion pressures below the normal autoregulating pressure range The increase in RBF induced by moderate doses of acetylcholine was nearly as large at low as at normal perfusion pressures In the study shown in Fig 5 RBF was 213 and 200 % of control values at perfusion pressures of 165 and 85 mm Hg respectively during infusion at a rate of 2 2 μg/min. With infusion at a rate of 10 μg/min RBF was 330 and 240 % of control flows at corresponding perfusion pressures A similar relationship between RBF and perfusion pressure was obtained in the 3 other dogs Proportional changes in RBF and perfusion pressure (Mahmod and Lanari 1964) were not observed in any experiment even at the highest rates of acetylcholine infusion

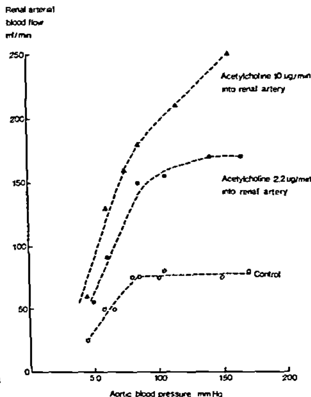


Fig 5 Effect of acetylcholine on pressure flow relationship

Effects of noradrenaline and angiotensin at normal and low perfusion pressures within the autoregulating range As autoregulating mechanisms might reduce the effects of vasoconstrictive agents by providing a counter-dilatation the effects on RBF of intra arterial injections of noradrenaline or angiotensin at normal perfusion pressure and at a perfusion pressure averaging 75 mm Hg were examined (Fig 6) At control perfusion pressure noradrenaline injections reduced RBF by 30—60 % and angiotensin reduced RBF by 40—80 % Reducing perfusion pressure to approximately 80 mm Hg lowered RBF by less than 5 % RBF was less reduced by angiotensin and noradrenaline at the low perfusion pressure On average the flow reducing effect of noradrenaline and angiotensin at low perfusion pressures was 65 and 76 % respectively of the effect at control pressure

Discussion

During i a infusion of noradrenaline and angiotensin at a rate reducing RBF to less than half of control values renal perfusion pressure could be reduced by 60 mm Hg or more before RBF dropped appreciably Similarly autoregulation was maintained during stimulation of the renal nerves and during intravenous infusion of angiotensin and noradrenaline These results are compatible with the assumption that

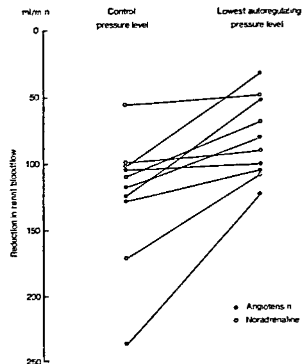


Fig 6 Reduction of renal blood flow following angiotensin and noradrenaline injections into the renal artery at control and lowest autoregulating pressure

renal autoregulation is a factor in circulatory homeostasis not only under resting conditions but also when the organism is subjected to strong humoral and nervous stimulation The present studies do not therefore concur with the view that renal autoregulation is overpowered by an increase in sympathetic discharge (Folkow and Langston 1964)

GFR may remain nearly unchanged when RBF is largely reduced by noradrenaline or angiotensin (Pitts 1963) As suggested by Green *et al* (1963), a constant glomerular capillary pressure and thereby constant GFR could be maintained if the vasoconstrictive effect of noradrenaline on the afferent arterioles was compensated by a counter-dilatation of the autoregulating elements According to their hypothesis the autoregulating elements would already be maximally dilated at a normal perfusion pressure This explanation is not supported by the observation that autoregulation was present to low perfusion pressures during infusion of vasoconstrictors Another argument against the hypothesis of counter-dilatation is that injections of noradrenaline and angiotensin produced similar or larger reductions in RBF at control pressures than at low perfusion pressures which give maximal autoregulatory vasodilatation It therefore seems that maintenance of GFR reflects a vasoconstrictive effect on both afferent and efferent arterioles so that glomerular capillary pressure remains constant A reduction in GFR does not necessarily imply reduced glomerular capillary pressure as colloid osmotic pressure rises as a consequence of increased filtration fraction

Our studies showed that RBF was maintained to lower perfusion pressures during angiotensin than during noradrenaline infusion It is possible that this difference reflects dissimilarity in the distribution of the vasoconstrictive effect between pre and post-glomerular vessels A more likely interpretation, however, is that the slight difference in lowest autoregulating pressure is caused by the different response to increments in concentration of angiotensin and noradrenaline When renal perfusion pressure was reduced below the autoregulating range, the vasoconstrictive effect of angiotensin was reduced or lost In contrast, the potency of noradrenaline was maintained As noradrenaline was infused into the renal artery, a reduction in RBF increased renal blood concentration The closing pressure (zero flow) was therefore considerably higher during noradrenaline infusion than during control experiments and during angiotensin infusion

Previous investigations of the effects of vasoconstriction on renal autoregulation are not directly comparable with the present studies Because isolation of the kidneys represents a large vasoconstrictive stimulus, RBF may, in isolated preparations, be more reduced during control periods than in the present studies during infusion of angiotensin and noradrenaline In our experiments, the efficiency of autoregulation was considerably higher than in isolated preparations, since RBF was reduced less than 5 % when perfusion pressure was reduced 50 mm Hg The distinction between presence and absence of autoregulation is therefore easier in dogs with intact circulation Another complicating factor in studies on isolated preparations is that the techniques employed for evaluating autoregulation often do not allow comparisons with other studies Waugh (1964) increased the perfusion pressure of isolated kidneys in a single step—from 100 to 190 mm Hg during angiotensin infusion, and from 100 to 160—180 mm Hg in control conditions—and claimed that angiotensin impaired autoregulation Noradrenaline has been found to abolish autoregulation (Folkow and Langston 1964, Waugh 1964), leave it unaffected (Nahmod and Lanari 1964), or re establish autoregulation in atonic kidneys (Schmid *et al* 1964) In the latter study a similar effect was demonstrated during angiotensin infusion. It was concluded that the return of autoregulation was due to increased tone of the vascular bed during angiotensin and noradrenaline infusion, suggesting that the presence of vasoconstrictors is indispensable This conclusion may not be warranted, however since the atonic kidneys had a perfusion pressure of 80 mm Hg whereas the renal perfusion pressure was well within the normal autoregulating range during infusion of noradrenaline and angiotensin

As the production of angiotensin is dependent on the release of renin from the juxtaglomerular granules in the wall of the afferent arterioles angiotensin might play a role in hemodynamic regulation Thurau and Schnermann (1965) and Thurau and co-workers (1967) proposed that angiotensin was responsible for autoregulation This hypothesis is not supported by the present studies, since autoregulation was not impaired when RBF was largely reduced by angiotensin infusion or when tachyphylaxis to angiotensin had developed

Vasodilating drugs such as procaine (Waugh 1958), papaverine (Thurau and

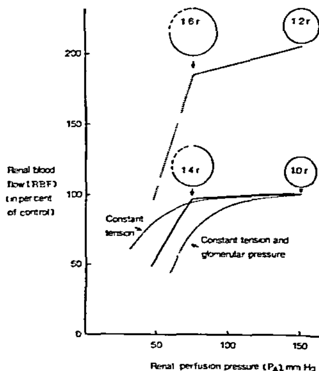


Fig 7 Flow/pressure relationship at normal and high rate of renal blood flow with corresponding changes in effective preglomerular radius according to Poiseuille's formula. The lower stippled curves are derived from Laplace's and Poiseuille's formulas (see text)

er 1959) and acetylcholine (Nahmod and Lanari 1964) have been found to abolish autoregulation in perfused preparations as the relationship between RBF and perfusion pressure became linear. It is not clear whether the curvilinear relationship shown in Fig 5 implies abolished autoregulation. Navar, Guyton and Langston (1966) obtained similar curves during infusion of hypertonic mannitol, and assumed that autoregulation was abolished. As apparent from Fig 5, RBF is much larger during acetylcholine infusion than under control conditions also at perfusion pressures below the lowest autoregulating pressure. GFR remains constant during acetylcholine infusion and is not essentially reduced by lowering perfusion pressure (unpublished observations). Maximal stretching of the elements participating in autoregulation cannot therefore account for the whole reduction in preglomerular resistance. On the contrary, vasoactive drugs might have no effect on the muscle elements participating in autoregulation.

Applications of Poiseuille's formula $P_A - P_G = k\,RBF/R^4$. P_A is arterial perfusion pressure, P_G glomerular capillary pressure and R effective preglomerular radius. If RBF and P_G remain constant when P_A is lowered from 150 to 75 mm Hg (autoregulation), R increases to 1.4R (Fig 7). In this calculation P_G is assumed to be 50 mm Hg. If it is higher, the increase in effective preglomerular radius would be more than 40 %.

This analysis is only valid if most preglomerular vessels react similarly during lowering of perfusion pressure. The evidence is twofold. First, by the use of the

hydrogen gas washout technique medullary blood flow has been found to be auto regulated (Aukland 1967) and blood flow in deep and superficial layers of the cortex shows a similar autoregulation pattern (Aukland and Loyning unpublished observa tions) Secondly, in most kidneys the distinction between presence and absence of autoregulation during lowering of the perfusion pressure is very sharp, suggesting that all preglomerular vessels react similarly

Post glomerular vessels do not participate in autoregulation as post glomerular resistance (P_G/RBF) cannot change as long as GFR and RBF remain constant As RBF increases and GFR (and therefore P_G) remain constant during infusion of acetylcholine, Poiseuille's formula can be used to calculate the increase in R Using the data shown in Fig 5, infusion of acetylcholine at a rate of 2 2 μg/min increases the preglomerular effective radius by 20 % (to 1 2R) Post glomerular resistance (P_G/RBF) is obviously halved by doubling RBF On the assumption that post glo merular resistance remains constant during subsequent lowering of perfusion pres sure, the increase in effective preglomerular radius caused by autoregulation can be calculated as previously It can be shown that the observed RBF at 75 mm Hg cor responds to an increase in effective preglomerular radius from 1 2R to 1 6R (Fig 7) The absolute increase in effective preglomerular radius (0 4R) is therefore as large as in control experiments Further, it can be demonstrated that P_G remains essential ly constant Autoregulation is therefore intact at moderate rates of acetylcholine in fusion At high rates of acetylcholine infusion (10 μg/min) the calculated radius is 1 35R at control pressure and 1 6R at low perfusion pressure The increase is accordingly only 0 25R, and it can be calculated that P_G is reduced and autoregu lation therefore no longer completely effective

The requirement that P_G remains constant during autoregulation implies either that the transmural pressure of the afferent arterioles also remains constant as suggested by Bayliss (1902) or that vascular tension T is the factor autoregulated According to Laplace s formula T = pR Thurau (1967) combined Laplace s and Poiseuille s formulas and stated that tension could not be kept constant during variation of perfusion pressure and accordingly was not the parameter autoregulated because constant tension would lead to decreasing flow at increasing perfusion pressure It is necessary however to integrate along the preglomerular arteriole If the pressure drop is dp along a segment of a preglomerular resistance vessel of length dl Poiseuille s formula can be written as

$$-dp - k_1 \, RBF \, R^{4} \, dl$$

The preglomerular resistance vessel can be divided into n segments each with different but constant tension T_1 T_n during variation of perfusion pressure Combination of Laplace s and Poiseuille s laws gives

$$\int_{P_A}^{P_G} -p^{4} \, dp - \int_{0}^{l} k_1 \, RBF \, (T_1^{4} + \quad T_n^{4}) \, dl$$

By integration of the equation and as $k_1 l (T_1^{4} \quad T_n^{4})$ is constant the relationship between arterial perfusion pressure (P_A) and RBF can be expressed as

$$RBF - k(P_G^{3} \quad P_A^{3})$$

where as previously P_G is glomerular capillary pressure Using this formula, the lower strippled curve in Fig 7 is obtained when P 50 mm Hg during lowering of P_A The upper stippled curve is obtained by using the less restrictive assumption that post glomerular resist ance P /RBF) remains constant By introducing the additional assumption that the behaves as a passive tube outside the range of ter cn regulation autoregulation en

to those obtained in experiments can be constructed This correspondence between hypothesis and observations obviously does not imply that tension is necessarily the factor controlled but simply states that the possibility has not yet been ruled out

According to this analysis the results obtained are compatible with the hypothesis that autoregulating elements sensitive to stretch are coupled around the circumference of the arterioles in series with contractile elements sensitive to vasoactive agents such as noradrenaline, angiotensin and acetylcholine, as shown in Fig 7 If two types of contractile elements exist, it would be expected that in certain experimental situations only the autoregulating elements would be dilated resulting in a moderate increase in RBF, whereas in other experimental situations both autoregulating elements and vasoactive elements might be dilated, resulting in a large increase in RBF Support for this view is presented in another paper (Kiil Kjekshus and Loyning 1969)

Independent operation of two types of contractile elements is also partly supported by other studies In the first place renin is released when blood pressure is lowered and the autoregulating elements are dilated A similar increase in effective preglomerular radius induced by vasodilating agents is not associated with renin release (Vander 1967) In the second place spontaneous and other changes in RBF and glomerular filtration rate at constant perfusion pressure are apparently caused by changes in the length of vasoactive and not in the length of autoregulating elements since autoregulation is present despite large variations in RBF and glomerular filtra[illegible]on rate

This study was supported by grants from the Norwegian Council for Science and the Humani[illegible]t es and the Norwegian Council on Cardiovascular Diseases We thank the Institute s Administrative Assistant Miss Aud Vogt for help throughout the work

References

AUKLAND K Stu [illegible] 3rd int Congr Nephro [illegible]

BAYLISS W M [illegible] essure J Physiol Lond [illegible]

FOLKOW B and J B LANGSTON The interrelationship of some factors influencing renal blood flow autoregulation *Acta physiol scand* 1964 *61* 165—176

GREEN H D C E RAPELA and M C CONRAD Resistance (conductance) and capacitance phenomena in terminal vascular beds In *Handbook of Physiology Section 2* Circulation Vol 2 *pp* 935—960 Amer Physiological Society Washington D C 1963

HERD J A and A C BARGER Simplified technique for chronic catheterization of blood vessels *J appl Physiol* 1964 *19* 791—792

KIIL F J KJEKSHUS and E LOYNING Role of autoregulation in maintaining glomerular filtration rate at large urine flow *Acta physiol scand* 1969 *76* 24—39

NAHMOD V E and A LANARI Abolition of autoregulation of renal blood flow by acetylcholine *Amer J Physiol* 1964 *20*[illegible] 1[illegible]3—12[illegible]

NAVAR L G A C GUYTON and J B LANGSTON Effect of alterations in plasma osmolality on renal blood flow autoregulation *Amer J Physiol* 1966 *211* 1387 139[illegible]

PITTS R F *Physiology of the Kidney and Body Fluids* 243 pp Year Book Medical publishers Inc., Chicago 1963

SCHMID H E JR R C GARRETT and [illegible] reduced renal pressure gradients *Circ* [illegible]

THURAU K Nature of autoregulation [illegible] *Washington 1966* Vol I pp 162— [illegible]

THURAU K and K KRAMER Weitere Untersuchungen zur myogenen Natur der Autoregulation des Nierenkreislaufes Aufhebung der Autoregulation durch muskulotrope Substanzen und druckpassives Verhalten des Glomerulusfiltrates *Pflugers Arch ges Physiol* 1959 *269* 77—93
THURAU K and J SCHNERMANN Die Natriumkonzentration an den Macula densa Zellen als regulierender Faktor fur das Glomerulusfiltrat (Mikropunktionsversuche) *Klin Wschr* 1965 *43* 410—413
THURAU K J SCHNERMANN W NAGEL M HORSTER and M WAHL Composition of tubular fluid in the macula densa segment as a factor regulating the function of the juxtaglomerular apparatus *Circulat Res* 1967 *20* (Suppl II) 79—89
VANDER A J Control of renin release *Physiol Rev* 1967 *47* 359—382
WAUGH W H Myogenic nature of renal autoregulation of renal flow in the absence of blood corpuscles *Circulat Res* 1958 6 363—371
WAUGH W H Circulatory autoregulation in the fully isolated kidney and in the humorally supported isolated kidney *Circulat Res* 1964 *15* (Suppl I) 156—169

Acta physiol. scand. 1969. 76. 24—39

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Role of Autoregulation in Maintaining Glomerular Filtration Rate at Large Urine Flow

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Received 6 July 1968

Abstract

KIIL F J KJEKSHUS and E LØYNING *Role of autoregulation in maintaining glomerular filtration rate at large urine flow* Acta physiol scand 1969 76 24—39

The effects of large urine flow and high tubular pressure on renal blood flow (RBF) and glomerular filtration rate (GFR) were studied in dogs In unanesthetized animals ethacrynic acid and furosemide infused during hypotonic saline or mannitol diuresis increased urine flow to more than half of the filtered load without reducing GFR, whereas clearance of paraaminohippurate (C_{PAH}) rose In anesthetized dogs ethacrynic acid and furosemide increased RBF By stepwise renal artery constriction RBF was reduced in proportion to perfusion pressure indicating abolished autoregulation A similar moderate increase in RBF and abolition of autoregulation were also obtained by ureteral obstruction In contrast autoregulation was maintained until RBF was nearly doubled during infusion of saline or mannitol During infusions of hypotonic solutions C_{PAH} increased despite reductions in serum osmolality which in renal perfusion experiments led to a fall in RBF

Conclusion High tubular or intestitial pressure acts on the autoregulating mechanism which dilates afferent arterioles until the hydrostatic pressure difference over the glomerular membrane is reestablished By this mechanism RBF is increased and GFR maintained at high urine flow Vasodilating factors may be elicited during expansion of extracellular volume but do not affect autoregulation and are without distinct effect on GFR.

With increased urine flow there is a rise in volume and interstitial pressure of the kidney The proximal tubular pressure may be more than 40 mm Hg during osmotic diuresis, according to micropuncture studies in rats (Gottschalk 1964) High tubular pressure would be expected to counteract glomerular filtration but as shown in the present studies a reduction in glomerular filtration rate (GFR) in dogs was not observed even when more than 50 % of the filtrate was excreted The mechanisms that maintain GFR and increase renal blood flow (RBF) during large urine flow, despite high tubular and interstitial pressures have not been adequately defined

The simplest assumption would be that the pressure drop over the glomerular membrane, the glomerular driving pressure remains constant because glomerular capillary pressure increases in parallel with tubular pressure An increase in glomeru

lar capillary pressure implies a reduction in preglomerular vascular resistance One possibility is that afferent arterioles are dilated by elongation of autoregulating elements As the interstitial pressure is similar to the proximal tubular pressure (Gottschalk 1964), the requirement of constant glomerular driving pressure implies constant transmural pressure of the afferent arterioles (Bayliss 1902) If this hypothesis were true autoregulating elements would be maximally dilated during large urine flow, and RBF would accordingly not be maintained when renal arterial perfusion pressure was lowered (abolition of autoregulation)

The alternative is that preglomerular vasodilatation induced by high urine flow does not interfere with autoregulation Acetylcholine infused into the renal artery may double RBF without seriously affecting renal autoregulation (Kiil, Kjekshus and Loyning 1969)

The purpose of the present studies was to examine the effects of potent diuretics such as ethacrynic acid and furosemide and of mannitol and saline infusion on GFR RBF and autoregulation If the increase in RBF could be accounted for by a maximal dilatation of autoregulating elements RBF would increase similarly whether tubular pressure was increased by high urine flow or by ureteral obstruction (Kiil and Aukland 1961) The effect of diuretics was therefore compared with the effect of raising renal pelvic pressure A few renal perfusion studies were performed to examine whether the increase in RBF observed during expansion of the extracellular fluid space with hypotonic solutions could be obtained by diluting renal arterial blood with hypotonic saline

Methods

Unanesthetized dogs Twenty-one clearance studies were performed in 10 trained female dogs weighing 21 to 24 kg in order to examine whether GFR could be reduced by increasing urine flow All dogs received 500 ml water by stomach tube half an hour before the experiments and intravenous infusion of inulin was started In some experiments paraaminohippurate (PAH) was infused after priming doses at rates ensuring optimal extraction Three clearance periods were obtained during intravenous infusion of 0 45 % NaCl at a rate of 8 ml/min In five studies ethacrynic acid (1 5 mg/kg b w) or furosemide (2 mg/kg b w) was then injected and 3—4 clearance periods obtained during infusion of maintenance doses of the diuretics and together with saline at rates corresponding to the urine flow In other studies urine flow was increased by increasing the rate of intravenous infusion of 0 45 % NaCl or 2 5 % mannitol to 30—40 ml/min and 4—7 clearance periods were obtained when urine flow had stabilized Ethacrynic acid or furosemide was then injected and 3—4 clearance periods obtained during the continued administration of mannitol and saline together with maintenance doses of the diuretics Urine was collected through an indwelling bladder catheter and venous blood specimens were obtained in the middle of each clearance period Inulin was determined by the method of Rolf Surtshin and White (1949) and PAH by the method of Smith *et al* (1945) GFR was measured as inulin clearance Sodium and potassium concentrations were determined by an Eel direct flame photometer and osmolality by an Advanced Instrument Osmometer

Anesthetized dogs Studies were performed on 10 dogs weighing 15 to 28 kg under intravenous sodium pentobarbital anesthesia (initial dose 30 mg/kg body weight) in order to examine renal autoregulation during various types of diuresis and ureteral pressures Parts of the technique were similar to that previously described (Kiil *et al* 1969) The left kidney was exposed through a flank incision Renal blood flow RBF was measured with an electromagnetic square wave flowmeter Nycotron Oslo with the flow probe placed on the renal artery close to the aorta In other studies the metered flow was compared with RBF measured by means of PAH-clearance and PAH-extraction A linear relationship was obtained over a wide range of RBF and the regression line used for determination of the calibrat

the flow probe A 20 gauge needle was inserted into the renal artery close to the kidney, and the perfusion pressure measured by a Statham transducer and recorded on a Sanborn recorder In some experiments, renal perfusion pressure was reduced by a plastic clamp on the renal artery between the flow probe and the 20 gauge needle The clamp was adjustable by a screw arrangement outside the body In other experiments renal arterial perfusion pressure was reduced either by constricting the aorta with a Blalock clamp, or by inflating a balloon in the aorta above the renal arteries In these experiments pressure was measured in the aorta below the renal arteries Ethacrynic acid or furosemide was injected into the renal artery at a dose of 1 5—2 mg/kg b w Intravenous infusions of saline or mannitol at high rates (up to 50 ml/min) were performed through a polyethylene catheter inserted into the vena cava from a femoral vein

Urine was collected by a polyvinyl catheter inserted into the renal pelvis through an incision in the upper part of the ureter Renal pelvic pressure might be kept at or below atmospheric pressure even during large urine flow, by lowering the distal end of the catheter Renal pelvic pressure was raised by clamping the catheter, or by elevating its distal end Pressure was measured through a needle inserted into the catheter close to the renal pelvis Urine from the other kidney was drained through a bladder catheter

Perfusion experiments designed to study the immediate vasomotoric effects of hypo , iso- and hypertonic solutions on renal circulation were performed on 4 dogs A wide bore cannula (outer diameter 5 mm) was tied into the carotid artery and connected by polyvinyl tubing to an extracorporeal flow probe which in turn was connected by polyvinyl tubing to the renal cannula with outer diameter 4 mm The system was primed with heparinized saline and the aorta exposed through a midline incision After insertion into the aorta a curve shaped renal cannula was advanced into the left renal artery RBF was interrupted less than 20 sec A solution of NaCl or mannitol was infused at a rate of 20 or 35 ml/min into the proximal part of the extracorporeal circulation by means of a constant delivery electric pump Perfusion pressure was measured through a needle inserted into the tubing close to the renal cannula from which blood samples for measurement of hematocrit and plasma sodium concentration were al o obtained Cooling of blood was avoided by immersing the extracorporeal system in a waterbath which together with the infusion fluids was maintained at 38° C Effects of hypo- iso- and hypertonic solutions on the vascular bed of the leg were studied in 4 dogs by a milar arrangement except that in some experiments blood was perfused by an electric pump rough a cannula tied into the proximal part of the femoral artery

Results

Effect of high urine flow on GFR in unanesthetized dogs The results of clearance studies in unanesthetized dogs are summarized in Fig 1 Each symbol represents the mean of 3—7 clearance periods Urine flow averaged 12 % of GFR during water diuresis and increased to 26 % of GFR during saline loading and to 36 % of GFR during mannitol infusion GFR increased on average from 76 to 87 ml/min during saline loading but was not significantly changed during mannitol diuresis Furosemide administered during water diuresis reduced GFR in 3 studies whereas etha crynic acid had no effect on GFR The diuretics had no consistent effect on GFR during saline and mannitol diuresis when urine flow rose to respectively 42 and 55 % of GFR.

PAH clearance (C_{PAH}) was determined during saline or mannitol loading and subsequent ethacrynic acid administration in studies on three dogs The protocol of one experiment is shown in Table I and the mean values for the clearance studies in each of the three dogs are presented in Table II During loading for more than an hour with hypotonic saline serum osmolality fell by 17—24 mOsm/kg H_2O and sodium concentration from an average of 144 to 134 meq/l A similar reduction in osmolality was observed during infusion of hypotonic mannitol, when serum sodium concentrations fell from an average of 141 to 110 meq/l PAH clearance increased similarly during saline and mannitol infusion, by an average of 27 %, and filtration

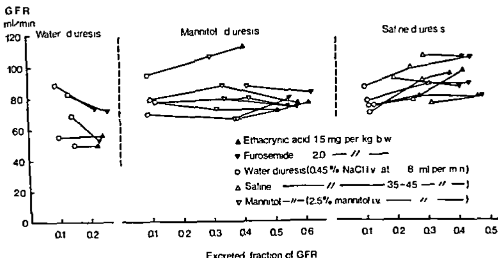


Fig. 1. Effect of high rates of urine flow on glomerular filtration rate (GFR). Ethacrynic acid or furosemide administered during water diuresis, mannitol and saline diuresis. Urine flow expressed as fraction of GFR.

fraction fell. After administration of ethacrynic acid, the increments in C_{PAH} were similar during hypotonic saline and mannitol diuresis, averaging 25 %.

Effects of ethacrynic acid and furosemide on RBF and autoregulation. The effects of ethacrynic acid on renal autoregulation were studied in five anesthetized dogs.

Recordings from one study are shown in Fig. 2. The left hand diagrams show renal perfusion pressure and RBF during mechanical constriction of the aorta at the height of diuresis. Renal autoregulation is abolished and there is no sign of overshoot of RBF after release of constriction. The recordings on the right were obtained during intravenous infusion of noradrenaline when urine flow was declining and show reappearance of autoregulation which is now present down to a perfusion pressure of approximately 100 mm Hg, i.e., 20—25 mm Hg higher than in control studies.

Fig. 3 shows flow/pressure relationships in the other four dogs. After control studies at low urine flow (0.5—1.0 ml/min) ethacrynic acid was administered into the renal artery. RBF rose slowly over several minutes and maximal rate was reached after 5—15 min concomitant with maximal urine flow which in these experiments varied between 11 and 16 ml/min. As shown in Fig. 3, RBF varied in proportion to perfusion pressure indicating abolition of autoregulation, and RBF was lower than in control studies at an arterial perfusion pressure corresponding to the lowest control autoregulating pressure.

When urine flow declined, autoregulation reappeared. In two of the dogs, the fluid loss after administration of ethacrynic acid was not replaced. Urine flow dropped to less than 5 ml/min despite continued administration of ethacrynic acid, and normal a

TABLE I Effect of hypotonic saline infusion and ethacrynic acid on plasma electrolyte concentrations and renal hemodynamics Excerpts of protocol in dog I

Time	Serum osmolality	Serum Na	Serum K	V	GFR	C_{PAH}
min	mOsm/kg H_2O	meq/l	meq/l	ml/min	ml/min	ml/min
0—			0 45 % NaCl at 8 ml/min			
20—30	283	146	3 9	6 2	73	237
30—40	283	145	4 0	6 8	72	232
40—50	282	144	4 1	7 2	73	240
55—			0 45 % NaCl at 35 ml/min			
135—145	266	134	3 7	22 2	81	311
145—153	264	133	3 6	23 4	82	318
153—161	262	132	3 5	23 6	79	309
162	Ethacrynic acid i v 1 5 mg/kg b w Infusion continued					
176—181	261	133	3 3	36 8	84	394
181—186	259	132	3 3	40 2	84	388
186—191	257	130	3 1	35 9	78	402

GFR inulin clearance C_{PAH} = para aminohippurate clearance

Autoregulation was also studied in two other dogs before and after administration of furosemide which increased urine flow to 18—20 ml/min In both dogs, RBF increased and autoregulation was abolished On average the diuretics increased RBF by 28 %

Effect of ureteral pressure The effect of high ureteral pressure on RBF was studied in three dogs at urine flows of 3—4 ml/min Urine flow was established by the infusion of 5 % mannitol In confirmation of previous studies (Kiil and Aukland 1961) *RBF increased when ureteral pressure was raised The highest obtainable* RBF ranged from 120—144 % of control values By raising the ureteral pressure in steps, a stepwise increase in RBF could be demonstrated, but the increase in RBF occurred more slowly and several minutes might elapse before a new equilibrium had been reached With further increase in ureteral pressure RBF finally declined

Gilmore's observations (1964) that RBF varied in proportion to renal perfusion prsesure at a ureteral pressure of 40—50 mm Hg were also confirmed Fig 4 shows the RBF/pressure relationship under control conditions and at two steps of increased ureteral pressure Autoregulation was absent whether ureteral pressure was kept constant by connecting the ureteral catheter to a reservoir of physiological saline, or was *allowed to fall In all experiments autoregulation reappeared* when renal pelvic pressure was lowered towards control values

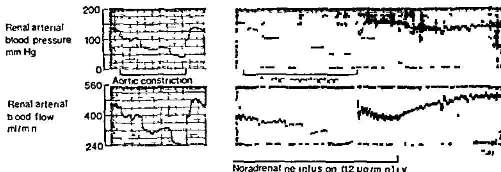


Fig 2 Recordings of perfusion pressure and renal blood flow showing abolition of autoregulation during ethacrynic acid administration at the height of diuresis (left) and reappearance of autoregulation at declining diuresis following noradrenaline infusion (right)

The similarity of the RBF pressure curves obtained during high ureteral pressure (Fig 4) and during administration of ethacrynic acid (Fig 3) or furosemide suggested that the same mechanism led to a moderate increase in RBF and abolition of autoregulation during stopped and high urine flow In support of this assumption, RBF fell when ureteral pressure was raised at high urine flows in contrast to the typical increase in RBF when ureteral pressure is raised at low urine flow (Fig 5)

Effects of saline and mannitol infusion The response to i v infusion of saline 0 9 % NaCl) at rates of 40—50 ml/min was investigated in six dogs after examination of renal autoregulation during control conditions RBF increased smoothly in

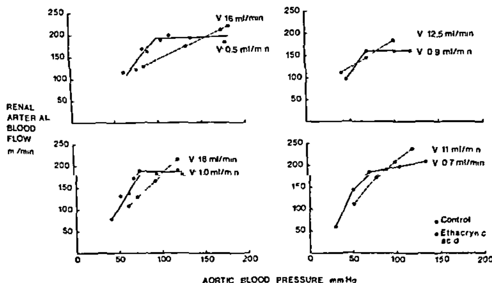


Fig 3 Abolition of autoregulation during high urine flow induced by ethacrynic acid Flow/pressure relationships during control conditions [illegible] and after [illegible] ethacrynic acid [illegible]

TABLE II Effect of hypotonic saline or mannitol infusion and ethacrynic acid* on serum solute

	Dog I			
	Serum osmolality mOsm/kg H_2O	Serum Na meq/l	GFR ml/min	C_{PAH} ml/min
Water diuresis	283	145	73	237
0 45 % NaCl	264	133	81	313
Ethacrynic acid	259	132	83	394
Water diuresis	279	139	76	240
2.5 % mannitol	266	117	74	310
Ethacrynic acid	258	109	72	373

* 0 45 % NaCl or 2 5 % mannitol was administered intravenously at 30—40 ml/min and etha

3 dogs and remained nearly unchanged in the other 3 for more than 20 min and then increased rapidly In contrast to the effect of ethacrynic acid the infusion of 500—1000 ml saline resulted in nearly as large an increase in RBF at low perfusion (80—90 mm Hg) as at normal prefusion pressure Renal autoregulation present until RBF was nearly doubled During continued infusion artery con triction resulted in proportional changes in perfusion pressure and RBF (Fig 6) But RBF continued to increase both at normal perfusion pressures and at pressures well below the normal range of autoregulation At the end of the infusion period—which always lasted more than one hour—RBF had increased by 100 to 220 % and urine flow ranged from 8 to 12 ml/min from one kidney Similar changes in the

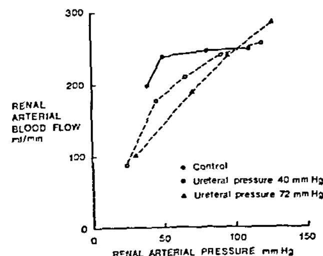


Fig 4 Relationship between renal blood flow and perfusion pressure before and after raising ureteral pressure

concentrations and on GFR and PAH clearance

Dog II				Dog III			
Serum osmolality mOsm/kg H_2O	Serum Na meq/l	GFR ml/min	C_{PAH} ml/min	Serum osmolality mOsm/kg H_2O	Serum Na meq/l	GFR ml/min	C_{PAH} ml/min
280	144	70	208	282	144	73	292
264	134	78	290	262	136	77	334
263	134	92	374	260	134	84	411
280	139	78	252	278	144	70	244
263	111	82	312	265	117	62	305
258	108	74	432	262	112	60	362

crynic acid in a dose of 1 5 mg/kg b w

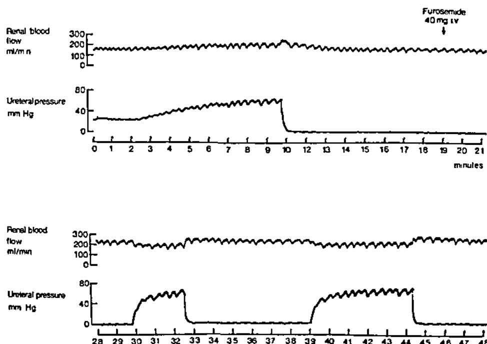


Fig 5 Effect of increasing ureteral pressure on renal blood flow at low urine flo curve) and high urine flow 11 min after furosemide administration (lower curve).

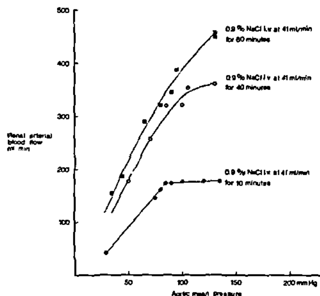


Fig 6 Relationship between renal blood flow and aortic mean pressure at various rates of i.v infusion of isotonic saline

relationship between RBF and perfusion pressure were also observed during infusion of hypertonic solutions (10 % mannitol and 2 % NaCl) at rates of 10—25 ml/min in studies on four dogs confirming the observations of Navar, Guyton and Langston (1966) made during infusion of hypertonic solutions in dogs

Perfusion studies In order to examine the direct effect of infusion of hypo- and hypertonic solutions at high rates on RBF, renal perfusion experiments were performed in 4 dogs The results are summarized in Table III A lowering of serum sodium concentration (and plasma osmolality) resulted in a decrease in RBF and an increase in serum sodium concentration in an increase in RBF The changes in RBF were not associated with significant changes in inulin clearance An increase in infusion rate from 20 to 35 ml/min of 0 82 % NaCl had little or no effect on RBF The flow response to isotonic solutions of NaCl and mannitol was not significantly different in spite of the much lower serum sodium concentration during mannitol infusion Occasionally during infusion of hypotonic solutions RBF became unstable with large regular fluctuations in RBF After stopping the infusion of hypotonic solutions a hyperemic period of several minutes duration usually followed Fig 7 shows data from all experimental periods in a dog during infusion of hypo- and hypertonic solutions of NaCl As apparent from the regression equation, RBF was reduced by an average of 15 ml/min by a reduction in serum sodium concentration of 10 meq/l

In perfusion studies on the hind leg similar results were obtained During perfusion at constant femoral blood flow the perfusion pressure was reduced during infusion of hypertonic solutions and increased during infusion of hypotonic solutions NaCl and mannitol in similar osmolal concentrations had the same effect on perfusion pressure

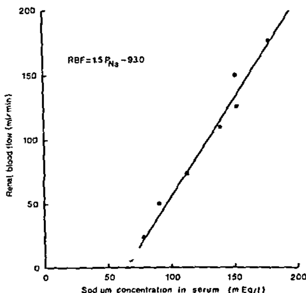


Fig. 7. Relationship between renal blood flow and serum sodium concentration in perfused kidney. Sodium concentrations were varied by infusing hypo- or hypertonic NaCl solutions at 20—35 ml/min into the plastic tube leading to the renal artery.

Discussion

The present studies showed that urine flow might be increased to more than half of the filtrate in unanesthetized dogs without reduction in GFR. The high urine flow was achieved by administering potent diuretics, such as ethacrynic acid or furosemide during hypotonic saline or mannitol diuresis. In those studies in which it was determined, PAH-clearance increased. A corresponding increase in RBF (20—30 %) was observed after infusion of ethacrynic acid or furosemide in anesthetized dogs. The increase in RBF was associated with abolition of renal autoregulation. A similar reduction in vascular resistance at normal arterial perfusion pressures combined with abolition of autoregulation was also obtained by increasing ureteral pressure. In contrast, a large increase in RBF during expansion of the extracellular volume was not associated with abolition of autoregulation. It seems, therefore, that different hemodynamic mechanisms are activated.

Hemodynamic effect of ethacrynic acid and furosemide. The moderate increase in RBF associated with abolition of renal autoregulation was probably not caused by a direct action of the diuretics on vascular smooth muscle or on some step in the autoregulating mechanism. The following evidence is in favour of this statement. a) Similar hemodynamic changes were induced by raising the ureteral pressure. b) The hemodynamic effects of furosemide and ethacrynic acid were less pronounced or absent in dehydrated dogs in which urine flow was only moderately increased. c) In hydrated dogs RBF increased slowly to a maximum in the course of several minutes, and maximal RBF coincided with maximal urine flow. In contrast, the in[crease in] RBF was immediate when [vasodilating agents such as acetylcholine and]

so [illegible] of [illegible] chloride were infused. The possibility that ethacrynic acid or [illegible] abolished autoregulation by some specific effect seems [illegible] to be ruled out.

The second hypothesis to examine is whether high intrarenal pressure, induced by [illegible] flow or ureteral obstruction, prevents vascular dilatation, thereby abolishing autoregulation. This hypothesis, however, does not explain why RBF [illegible]creases at normal perfusion pressure when tubular pressure is raised.

The third hypothesis is that an increase in intrarenal pressure reduces preglomerular vascular resistance so that the pressure drop over the glomerular membrane remains constant. This hypothesis is compatible with all findings in the present study. According to this view, abolition of autoregulation implies activation to a maximum so that the preglomerular arterioles are dilated as much as they can be in the [illegible] of autoregulation. But as discussed later, other stimuli may dilate them further.

This hypothesis also implies that it is the same stimulus which abolishes autoregulation whether intrarenal pressure is raised or arterial perfusion pressure reduced. However, maximal dilatation of autoregulating elements, as when [illegible] at low urine flow arterial perfusion pressure is reduced from 1[illegible] to 7[illegible] mm Hg, implies a reduction in total vascular resistance to half of control values. It may therefore be asked why RBF is not doubled instead of being increased by 20–30% by raising intrarenal pressure. The probable answer is that an increase in renal interstitial pressure by reducing the effective driving pressure counteracts an increase in RBF.[1] Tubular pressure [illegible] increased both by ureteral obstruction and by large urine flow [illegible] small transtubular gradients can be held, an increase in proximal tubular pressure implies a general increase of intrarenal pressure (Gottschalk 19[illegible]). The pressure RBF-reducing effect is probably not due to a uniform reduction of vascular diameters on account of high outside pressure, but is better explained in terms of dynamics of collapsible vessels as outlined by Permutt and Riley (1963). When the intrarenal pressure is increased by high urine flow or by ureteral obstruction and exceeds the venous outflow pressure, small veins will partly collapse. Proximal to the site of the collapse the pressure in the veins will be nearly similar to the intrarenal pressure. [illegible] a result of this mechanism the pressure difference between the [illegible] and the [illegible] may be considerable even though there is no [illegible] total [illegible]. The driving pressure for flow through the kidney is [illegible] more reduced [illegible] the difference between arterial perfusion pressure and intrarenal pressure [illegible] the difference between arterial and venous pressure [illegible] suggest.

The following arguments [illegible]

[1] [illegible] pressure [illegible] mm Hg and a mean arterial perfusion pressure of [illegible] mm Hg [illegible] RBF [illegible] pressure of [illegible] mm Hg [illegible] 13 [illegible] RBF [illegible] intrarenal pressure [illegible] RBF. The increase [illegible] pressure.

tion by raising intrarenal pressure and by lowering arterial perfusion pressure is the same. a) When autoregulation was abolished by increasing ureteral pressure, the effect of lowering arterial perfusion pressure was a reduction in RBF. b) Conversely, when autoregulation was abolished by lowering the arterial perfusion pressure, the effect of raising ureteral pressure was a reduction in RBF.

Bayliss (1902) suggested that the factor autoregulated by lowering of arterial perfusion pressure was the transmural pressure at the glomerular end of the afferent arterioles. More recent investigators have felt that maintenance of the composition of tubular fluid in the macula densa region was a more attractive alternative. As previously shown (Kiil *et al.* 1969), we have no possibility of dissociating between tension and pressure as the factor controlled. In the following the hypothesis is called the transmural pressure hypothesis. The implications of this hypothesis are that both an increase in interstitial pressure and a reduction in arterial perfusion pressure result in a reduction in preglomerular resistance, so that the transmural pressure at the end of the afferent arterioles remains constant. The effect of increasing interstitial pressure at normal and constant arterial perfusion pressure would therefore be an increase in RBF and nearly unchanged GFR, since the hydrostatic pressure difference over the glomerular capillaries remains constant. By raising ureteral pressure Kiil and Aukland (1961) found that RBF increased and GFR remained constant up to a ureteral pressure of 50—60 cm water. The present studies showed that autoregulation was abolished by increasing ureteral pressure by 40—60 mm Hg. As a lowering of arterial perfusion pressure by 50—60 mm Hg is required at normal ureteral pressure before autoregulation is abolished, the range of compensation by autoregulation seems to be similar whether perfusion pressure is lowered or tubular pressure raised. If the tubular and interstitial pressure could be increased beyond autoregulatory compensation, by increasing urine flow, a reduction in RBF would be expected, as observed when ureteral pressure is sufficiently raised. However, in our experiments, RBF never fell when urine flow was increased. A possible explanation is that an increase in interstitial pressure which reduces RBF and GFR also reduces the flow rate through the tubules with subsequent reduction of interstitial pressure. By this mechanism abolition of renal autoregulation would limit further increase of urine flow.

The increase in tubular pressure at high urine flow may be caused either by intrarenal resistance mainly localized in the collecting ducts (Gottschalk 1964), or by extrarenal resistance that increases the pressure in the renal pelvis. In the present studies renal autoregulation was examined in kidneys with drained renal pelvis, using an arrangement which allowed pelvic pressure to be kept close to atmospheric pressure even at the highest obtainable urine flow. The abolition of autoregulation after furosemide and ethacrynic acid administration was therefore not due to an increase in renal pelvic pressure. In the intact urinary tract, however, an increase in renal pelvic pressure may contribute significantly to the increase in tubular pressure. Intrapelvic pressure may transiently increase to more than 50 mm Hg after ethacrynic acid administration but is reduced to 10—20 mm Hg in the course of a few minutes,

on account of compensatory changes in the dynamics of the ureter (Kiil and Kjekshus 1967) An increase in bladder pressure or partial obstruction of the intramural part of the ureter during distension of the bladder is not compensated for, however during large urine flow The renal pelvic pressure may therefore be 30—50 mm Hg at full bladder during ethacrynic acid diuresis

The demonstration in isolated preparations that the intrarenal pressure increases with arterial pressure (Hinshaw 1964), suggests that renal autoregulation in the isolated kidney is mainly due to the waterfall effect Active autoregulating mechanisms are apparently extinguished as an increase in interstitial pressure would tend to increase RBF Studies of autoregulation in isolated preparations should therefore be discontinued if it cannot be shown that RBF increases by raising the ureteral pressure

Saline and mannitol diuresis Considerable increments in RBF induced by intravenous administration of saline or mannitol were not associated with an abolition of renal autoregulation In conformity with this observation most studies showing abolition of autoregulation by raising ureteral pressure have been performed under conditions of mannitol diuresis (Kiil and Aukland 1961 Gilmore 1964 Hårsing Szanto and Bartha 1967) In our studies RBF increased during expansion of the extracellular fluid volume both at normal and low renal perfusion pressures The reduction in renal vascular resistance could not therefore be explained by dilatation of autoregulating elements On the assumption that glomerular capillary pressure remained constant calculations based on Poiseuille's law as previously outlined (Kiil *et al* 1969) showed that the increase in effective glomerular radius induced by lowering perfusion pressure was similar before and during saline loading until RBF was doubled Saline and mannitol infusion therefore seem to have the same effect on renal hemodynamics as acetylcholine and hypertonic solutions of mannitol and saline The action of these vasodilators is mainly on smooth muscle elements coupled in series with the autoregulating elements around the circumference of the preglomerular vessels (Kiil *et al* 1969) In similarity with vasoactive agents such as acetylcholine angiotensin and noradrenaline no significant changes in GFR occurred during considerable changes in RBF induced by varying the tonicity of the perfusate (Table III)

The effect of high intrarenal pressure on autoregulation is not altered As apparent from Fig 5 RBF varies in proportion to perfusion pressure after large expansion with saline The calculated effective preglomerular radius has then increased by 55 %, and is similar at all perfusion pressures The relationship between urine flow and interstitial pressure however may be different during ethacrynic acid and saline diuresis as the collecting ducts may be more dilated during a slowly rising diuresis The renal pelvis and ureter are more dilated when the pressure is raised over a longer period (Kiil and Kjekshus 1967) Reduction of nephron resistance would obviously contribute to the maintenance of autoregulation during large urine flow

Besides abolition of autoregulation at least 3 factors might contribute to the in

TABLE III Effect of infusion of iso- and hypotonic solutions of NaCl into shunt to left renal artery

		PP mm Hg	RBF ml/min	C_{IN} ml/min	P_{Na} meq/l	Hct
Dog 1						
	Control	135	160	46	139	35
	0.75 % NaCl at 20 ml/min	155	110 (90)	44	133	28
Dog 2						
	Control	—	160	25	143	37
	0.82 % NaCl at 35 ml/min	—	180 (145)	24	146	23
Dog 3						
	Control	125	165	33	137	25
	0.75 % NaCl at 20 ml/min	125	125 (105)	33	129	17
Dog 4						
	Control	105	135	—	140	35
	0.9 % NaCl at 35 ml/min	105	165 (130)	—	146	26

PP — Perfusion pressure measured at the end of the shunt where serum sodium concentration (P_{Na}) and hematocrit (Hct) were determined. RBF is the total flow rate. Number in parenthesis is blood flow when flow of infusate has been substracted.

crease in RBF during loading with saline and mannitol. First, as shown in Fig. 6, RBF is dependent on the osmolality of the perfusate. Secondly, reduction in viscosity might increase RBF. The perfusion experiments suggest that this factor is of minor importance. Thirdly, vasodilating substances might be released during expansion of the extracellular fluid volume. C_{PAH} increased by an average of 27 % during infusion of hypotonic saline and mannitol (Table II). With intravenous infusion of hypotonic saline serum sodium concentration was reduced by an average of 11 meq/l. The direct effect on the renal vessels of such a reduction in serum sodium concentration would—according to Fig. 7—be a reduction in RBF of approximately 17 %. Most of the flow-increasing effect of hemodilution has been accounted for by this figure, as the perfusion experiments were performed by infusing solutions at a rate of 35 ml/min, whereas control RBF at normal serum sodium concentrations was measured during periods without infusion. The opposite directional changes in RBF of i.a. and i.v. infusions of hypotonic saline suggest that a vasodilating factor is elicited by expansion of the extracellular fluid volume. As no constant relationship was observed between amounts of saline infused intravenously and changes in RBF, the release of any such hypothetic factor is highly variable.

Several factors besides autoregulation might also participate in the regulation of GFR. Hemodilution by reducing the colloid osmotic pressure would increase GFR at constant glomerular transcapillary pressure. As apparent from Table III, GFR was not significantly changed when large variations in RBF were induced by varying the

tonicity of the perfusate GFR usually increased during saline but remained constant during mannitol infusion (Fig 1 Table II) It has previously been demonstrated that amino acids such as glycine may increase GFR more than expansion of extra cellular volume and with little or no effect on renal sodium excretion (Kiil 1965) Our studies do not indicate that such factors—which mainly reduce preglomerular vascular resistance are important in the regulation of GFR at high urine flow We therefore conclude that the maintenance of GFR at high tubular pressure during high urine flow is mainly caused by autoregulating mechanisms

Mechanisms of autoregulation Due to their strategic localization the juxtaglo merular and the *macula densa* region of the distal tubules have been considered as the site of autoregulation The factor regulated has been assumed to be the osmolal ity (Navar *et al* 1966) or the sodium concentration of the tubular fluid passing the macula densa region (Thurau and Schnermann 1965)

The observations that renal autoregulation can be abolished both by large urine flow and by ureteral obstruction are difficult to reconcile with these hypotheses Ethacrynic acid inhibits sodium reabsorption out of the ascending limb of Henle s loop (Goldberg *et al* 1964) and the tubular fluid delivered to the macula densa *region is therefore nearly isotonic with plasma During ureteral obstruction sodium* concentration and osmolality of the tubular fluid at the macula densa region would if anything be lowered An increase in sodium concentration or osmolality seems therefore not to be a necessary requirement for abolition of renal autoregulation

The present studies strongly support the transmural pressure hypothesis Control transmural pressure or vascular tension might be a general property of arteriolar muscles since autoregulation is present in organs other than the kidney As the autoregulation is more complete in the kidney more specialized structures—such as the juxtaglomerular apparatus— might participate in renal autoregulation The present study is compatible with the hypothesis that the factor controlled is the pres sure difference between afferent arterioles and distal tubules across the macula densa

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References

BAYLISS W M On the local reactions of the arterial wall to changes in internal pressure *J Physiol* (Lond) 1902 *28* 220—231

GILMORE J P Influence of tissue pressure on renal blood flow autoregulation *Amer J Physiol* 1964 *206* 70 713

GOLDBERG M D K MCCURDY E L FOLTZ and L W BLUEMLE JR Effects of ethacrynic acid (a new saluretic agent on renal diluting and concentrating mechanisms Evidence for site of action in the loop of Henle *J clin Invest* 1964 *43* 201—215

GOTTSCHALK C W Micropuncture measurements of intrarenal pressures *Circulat Res* 1964 *15* (Suppl I) 110—114

HARSING L G SZANTO and J BARTHA Renal circulation during stop flow in the dog *Amer J Physiol* 1967 *213* 937—938

HINSHAW L B Mechanism of renal autoregulation role of tissue pressure and description of a multifactor hypothesis *Circulat Res* 1964 *15* (Suppl I) 120—129

KIIL F The effects of increased glomerular filtration rate and of extracellular volume expansion on tubular sodium reabsorption *Proc Internat Union of Physiol Sciences* 1965 5 171
KIIL F and K AUKLAND Renal concentration mechanism and hemodynamics at increased ureteral pressure during osmotic and saline diuresis *Scand J clin Lab Invest* 1961 *13* 276—287
K[illegible]
K[illegible]
[illegible]
on renal blood flow autoregulation *Amer J Physiol* 1966 *211* 1387—1392
PERMUTT S and R L RILEY Hemodynamics of collapsible vessels with tone the vascular waterfall *J appl Physiol* 1963 *18* 924—932
ROLF D A SURTSHIN and H L WHITE A modified diphenylamine procedure for fructose or inulin determination *Proc Soc exp Biol (N Y)* 1949 *72* 351—354
SMITH H W N FINKELSTEIN L ALIMINOSA B CRAWFORD and M GRABER The renal clearances of substituted hippuric acid derivatives and other aromatic acids in dog and man *J clin Invest* 1945 *24* 388—404
THURAU K and J SCHNERMANN Die Natriumkonzentration an den Macula densa Zellen als regulierender Faktor fur das Glomerulusfiltrat (Mikropunktionsversuche) *Klin Wschr* 1965 *43* 410—413

Acta physiol. scand 1969 76 40—48

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A Comparative Study of K^{42} and Na^{24} Movements during the Cardiac Cycle

By

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Received 16 July 1968

Abstract

Coraboeuf E J Delahayes and U Sjostrand *A comparative study of K^{42} and Na^{24} movements during the cardiac cycle* Acta physiol scand 1969 76 40—48

Studies of K^{42} and Na^{24} movements during the cardiac cycle were made on right ventricle from the rat and guinea pig on frog atrium and on frog ventricular strips The methods used in these studies have been described in earlier papers cited in the text The K^{42} study showed a cyclic leakage of radioactive potassium from the rat ventricle no such leakage was observed ventricle from the guinea pig Both frog preparations showed an increase of K^{42} efflux ng the electrical activity The Na^{24} study showed a slight decrease in the radioactive sodium efflux from the rat and guinea pig ventricle at the depolarization phase and the first part of the action potential in frog preparations a large increase in the activity efflux preceded by a slight decrease was observed The possibility of mechanical artifacts is discussed and an interpretation of the results is proposed which is based on a different pattern of potassium permeability between the species the guinea pig and frog showing a rectangular action potential and the rat a "sigmoid action potential The slight decrease in Na^{24} activity preceding the mechanical systole could reflect the basic ionic mechanisms of the depolarization

Previous findings (Wilde and O Brien 1953 Danielson Öbrink and Sjostrand 1962, Lerber *et al* 1962) of cyclic leakages of K^{+} and Na^{24} during the cardiac activity have recently been questioned (Sjostrand 1964 Pak *et al* 1966) and it has been suggested that such leakages could result from the mechanical activity either by a squeezing effect or by a change of th area/volume ratio of the preparation when it contracts Other investigations however have shown differences in the pattern of the potassium movement during the cycle according to the species studied *e g* decreased leakage in the frog (Lamb and McGuigan 1966) increased leakage in the rat and no systolic change in the guinea pig (Delahayes and Coraboeuf 1967)

The aim of the present study was to ascertain whether the observed cyclic leakages might have a physiological origin other than a simple squeezing effect

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Methods

The experiments were carried out (i) on rat and guinea pig right ventricle perfused through the coronary arteries and (ii) on two types of frog heart preparations, *i.e.* atrium and ventricular strips In the experiments on frog atrial preparations we used the method previously described by Danielson, Öbrink and Sjöstrand (1962) and Sjöstrand (1964) while in the experiments on ventricular strips from the frog rat and guinea pig the method described by Delahayes and Coraboeuf (1967) was used (modified in the case of frog strips because of the smaller size of the preparation and the absence of coronary vessels)

The activities of the collected samples were counted by means of a gamma spectrometer corrected for the radioactive decay and calculated per weight unit Each sample activity was expressed in per cent of the mean activity of the perfusate collected in the first sample tube of the collector The results of the double tagging experiments on the frog atrium (calculated according to the method described by Öbrink and Ulfendahl (1959) with technical and practical aspects taken into account in accordance with Danielson *et al* (1966)) were plotted directly on the curve

For the K^{42} continuous efflux study the method described by Schreiber (1956 see also Revial *et al.* 1965) was used and adapted to the ventricular strip The strip perfused through its coronary arteries is placed in a small chamber and washed continuously by a flow of non radioactive solution maintained at a constant level by suction. The strip is stimulated by two electrodes and the contraction recorded by means of a strain gauge The chamber is lowered into the well of a gamma spectrometer This set up allows recordings of the loading and unloading K^{42}-curves of the strip

The physiological solutions saturated with O_2 (frog) or with a mixture of 97 % O_2 and 3 % CO_2 (rat and guinea pig) had the following compositions

Frog heart 6 5 g NaCl (111 2 mM) 0 2 g KCl (2 68 mM) 0 2 g $CaCl_2$ 1 80 mM), 0 1 g $NaHCO_3$ (1 19 mM and 1 g glucose per litre (pH corrected to 7 2)

Mammalian heart 7 6 g NaCl (131 mM) 0 42 g KCl (5 6 mM) 0 24 g $CaCl_2$ (2 16 mM) 1 g $NaHCO_3$ (1 19 mM 0 05 g $MgCl_2$ (0 24 mM and 2 g glucose per litre (pH corrected to 7.35

The radioactive solutions had the same composition but in those using K^{42} all K in the solution came from irradiated material and in those using Na^{24} 2 to 8 mM Na^{24} Cl were simply added to the inactive solution The specific activities were 6 10^{3} C/g Na and 4 10^{7} C/g K

The experiments were carried out at a temperature of 24 C in the case of the rat and guinea pig and at 18 C Fig 3 and 7 5° C Fig 4) in the case of the frog

Results

Cyclic effluxes

Fig 1 shows the results of 4 expts performed on guinea pigs and 4 performed on rats using K^{42} The preparation was stimulated (vertical arrow) 350 msec after the beginning of the first sampling (zero time No alteration in radioactivity was observed in the guinea pig heart during the cycle while a net increase followed the cellular activation in the rat The time required for the solution to cross the perfusion system led to a delay in the action potential by about 100 msec in respect to the stimulation time The duration of the radioactivity increase did not appear to correspond to the duration of the tissue K^{42} leakage Short injections (30 msec) of radioactive solution into the aorta of non loaded strips gave peaks decreasing with a time constant of about 300 to 350 msec a value close to that observed in Fig 1 (lower tracing

Fig 2 refers to the study of Na^{24} movements in rat and guinea pig hearts under the same conditions as described above No increase in the Na^{24} leakage was observed after the depolarization in either the rat or the guinea pig On the contrary the stimulation was followed by a slight decrease in radioactivity

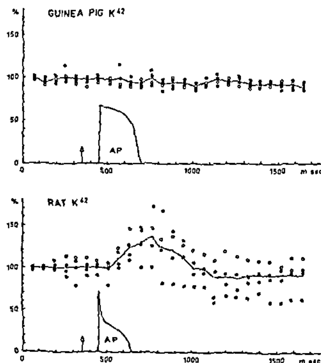


Fig 1 K^{42} leakage during the cardiac cycle in the guinea pig and rat Results of four experiments are indicated by different circles in both cases The mean values are plotted on a continuous tracing Sample activity is expressed in per cent of the mean activity of the perfusate in the first sample tube Time of stimulation is indicated by a vertical arrow The corrected position of the action potential is shown schematically

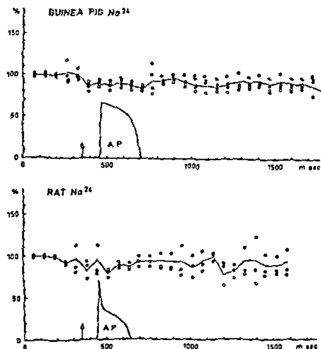


Fig 2 Na^{24} leakage during the cardiac cycle in the guinea pig and rat The results are plotted as described in Fig 1

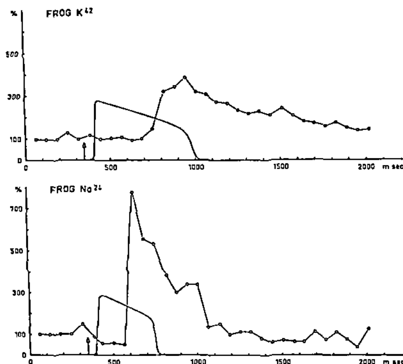


Fig 3 K^{42} leakage (above) and Na^{24} leakage (below) during the cardiac cycle in frog ventricular strips The results are plotted as described in Fig 1

With the same set-up as for the rat and guinea-pig a series of control experiments on frog ventricular strips was performed (Fig 3), which confirmed the results of earlier studies mentioned above, namely the development of a peak of K^{42} and of Na^{24} the latter being of greater amplitude than the former and preceded by a slight decrease

Double tagging experiments carried out on spontaneously beating frog atria showed rhythmic leakages of Na^{24} and K^{42} both peaks were practically synchronous with the atrial activity In the experiment in Fig 4 the atrium was loaded with an active solution containing 14 3 kcpm Na^{24}/mg and 5 4 kcpm K^{42}/mg i e $Na^{24}/K^{42} =$ 2 63 (1 kcpm = 10^3 cpm) After the efflux experiment, the total amounts of Na^{24} and K^{42} in the preparation were 431 kcpm and 304 kcpm, respectively (total Na^{24}/total K^{42} = 1 33) although the efflux of K^{42} was very small during this experiment A similar result for double tagging experiments on spontaneously beating frog atrium was shown for Na^{24} and Br^{82} (Br was used as a "Cl tracer") by Sjöstrand (1964)

Continuous efflux of K^{42}

The divergent results concerning K^{42} leakage during the cardiac cycle in and guinea pig (possibly explainable by differences in the pattern of the

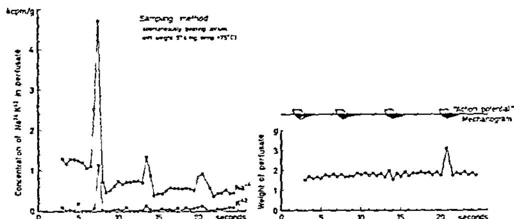


Fig. 4. The effluxes of Na^{24} and K^{42} from a spontaneously beating frog atrium. The figure shows the concentrations of Na^{24} and K^{42} in the perfusate samples (sample volume shown below; two samples per second). The electrical and mechanical activities are shown schematically.

permeability to potassium ions in the two species) initiated further studies of the K^{42} efflux kinetics on preparations previously loaded with this isotope. The data in *Table I* show that strips stimulated at a frequency of 60 pulses per minute had significantly different efflux constants: T 1/2 = 26 min in the rat, T 1/2 = 43 min in the guinea-pig (P = 0.001).

Discussion

The above results show (1) the occurrence of a cyclic increase in the K^{42} leakage from rat and frog hearts and a complete absence of such a phenomenon in guinea-pig hearts; (2) a very marked increase in the Na^{24} activity in frog hearts (preceded by a slight decrease) and the absence of such an increase in rat and guinea-pig hearts while the initial decrease occurred here also. It is difficult to explain these phenomena entirely by a squeezing effect due to cardiac contraction. Sjöstrand (1964) discussed different possible "squeezing" effects in frog myocardium and concluded from experimental findings that deformation of the extracellular space was highly improbable as the cause of Na^{24} and Br^{82} patterns in relation to the electromechanical cycle of the heart. On the other hand it was pointed out that the pos-

TABLE I. Mean T 1/2 values of intracellular K^{42} efflux from guinea pig myocardium (5 expts.) and from rat myocardium (6 expts.). (P = 0.001)

Species	T 1/2 values of intracellular K^{42} efflux	Mean
Guinea pig	35 min 31 min 42 min 38 min 50 min	43 min
Rat	30 min 26 min 28 min 23 min 23 min 24 min	26 min

sibility of altered physico-chemical properties as the result of mechanical distortion of the extracellular matrix during the cardiac cycle could not be disregarded In deed it is unlikely that mechanical activity would produce a K^{42} leakage in the rat without producing any in the guinea pig Though the contraction of the rat heart is stronger than that of the guinea pig heart the more spongy structure of the guinea pig heart should partially compensate this difference and render unlikely the complete suppression of a squeezing effect in this species On the other hand recent experiments have shown that in the rat a part of the perfusate radioactivity in crease could be due to a mechanical artifact, in these experiments the addition to K^{42} physiological solution of small amounts of sodium sulphate tagged with S^{35} of high activity led to the development of a systolic S^{35} peak but of smaller amplitude than the potassium peak (the ratio of the increase in K^{42} and S^{35} activity ranging from 1/3 to 1/2) Therefore it seems that a mechanical artifact cannot alone explain the whole of the observed K^{42} efflux The results illustrated in Fig 4 and those obtained by Danielson Öbrink and Sjostrand (1962) on frog atrium show that the Na^{24} leakage is much more marked than the K^{42} leakage though the tissue K^{42} activity remains high throughout the efflux experiment suggesting that cyclic potassium efflux could in fact be an artifact in this species The Br^{82} leakage (Br^{82} used as tracer for Cl leakage) on the other hand shows in spontaneously beating frog atrium a different efflux constant from Na^{24} but Br^{82} efflux shows cyclic variations indistinguishable from Na^{24} efflux cycles (Sjostrand 1964) If this is accepted frog and guinea pig myocardium should not produce any systolic cellular potassium leakage while such a leakage could occur in rat myocardium This dif ference in behaviour could be explained by different patterns of potassium perme ability during systole in the rat on the one hand and in the guinea pig and frog on the other hand Further at 24° C the rat action potential with its sigmoid shape is different from that of the guinea pig and frog which is rectangular (Coraboeuf Kayser and Gargouil 1956) In these two latter species an important decrease in the potassium permeability could occur at the beginning of the plateau (anomalous rectification Coraboeuf *et al* 1958 Brady and Woodbury 1960 Hutter and Noble 1960 In the rat this decrease could be less marked this is suggested by a recent study concerning the effects of tetraethylammonium (T E A) an inhibitor of potas sium permeability on the electrical activity of rat and guinea pig heart (Coraboeuf and Vassort 1967) This hypothesis is strengthened by the data of Table I which show a weaker potassium permeability in guinea pig ventricular strips than in those of the rat when both are stimulated at 60 pulses per min It may also be noted that the absence of K^{42} leakage in rabbit papillary muscle mentioned by Pak *et al* (1966) agrees with the present result in the guinea pig these two species having the same electrical activity (rectangular action potential)

As far as the Na^{24} experiments are concerned the absence of sodium leakage in the rat and in the guinea pig provides some support for the hypothesis that the squeezing effect could be very weak in the mammalian ventricular strip perfused through coronary vessels On the other hand this effect should be greater in the

case of the frog myocardium because of its sponge like structure However in the frog the increase of Na^+ activity in the perfusate is usually preceded by a slight decrease similar to that observed in the mammalian heart This decrease might reflect the rapid sodium influx occurring during the depolarization (Hodgkin and Huxley 1952 Weidmann 1956 Coraboeuf *et al* 1958 Noble 1962) by possible partial extracellular reabsorption of Na^+ during gNa increase It is of interest to note that this decrease in Na^+ efflux shown in Fig 2 and also detectable in Fig 4 and already described in the literature is synchronous with the very beginning of the depolarization or even seems to precede it In the case of Fig 2 this may be explained by *the uncertainty in the position of the beginning of the depolarization in* relation to the measured radioactive tracer pattern already discussed by Delahayes and Coraboeuf (1967) If this is true the rapid sodium influx occurs at the depolarization time or even precedes it as indicated experimentally for Na^{24} in the *spontaneously* beating frog sinus venosus (Sjostrand 1964) These results might therefore possibly not be mechanically generated artifacts (in the frog where the squeezing effect may be of greater amplitude the decrease in Na^{24} efflux may be partly obscured by a mechanical artifact while in the rat where the depression phase is less marked than in the guinea pig the weak squeezing effect could contribute to cancelling the phenomenon that would appear more clearly in the guinea pig heart because of its weaker contraction)

The diminished Na^+ efflux in Fig 2 clearly precedes the contraction of the cardiac tissue It is very tempting to regard this as a mirror image of Sjostrand s (1964) results for the Na^+ influx pattern obtained in two spontaneously beating frog sinus venosi A somewhat different pattern for Br^{S} was also obtained in another successful experiment By different experimental procedures Sjostrand failed to explain the Na^+ and Br influx patterns as being artifacts and it seemed highly possible therefore that the results obtained could in fact reflect the basic ionic mechanisms of the preparation

All experimental data accumulated in the literature regarding ionic fluxes during the cardiac cycle seem for the moment—like the results presented in this paper—to be in not very good agreement This could be due either to real differences in the biophysical processes or to differences as regards artifact producing mechanisms Recent theoretical considerations by Teorell (1968) of possible mechanisms for oscillatory water and net ionic fluxes (denoted parametric ionic pumping) in wet metastable physical systems could shed some light on these problems It has been shown by Teorell (1968) by measurements on the artificial membrane oscillator (se Teorell 1959) that when the system oscillates in an apparently undamped fashion the net flows of water and two ions (with different mobilities) have the same direction at the depolarization and early plateau phases of the membrane potential In the case of highly damped oscillations or in the metastable quiescent state *i e* resting potential *the contribution of the bulk flow of water* causes the ions to have separate net flow patterns during the abovementioned phases of the membrane potential The net flow of ions could also be changed by this means in such a way

that the net fluxes have opposite directions—If these results are also valid for biological systems like cardiac cell membranes they would implicate that spontaneously beating and electrically stimulated preparations could give different results although the basic mechanisms of function are very similar (and for the same reasons net ionic fluxes measured in 'voltage clamped' membrane systems will not be directly comparable with results presented in this and similar studies)

Conclusions

Our experiments showed a leakage of K^{42} during the cardiac activity in the rat at 24° C No similar phenomenon was seen in the guinea pig Concerning the Na^{24} leakage these two species showed no cyclic increase but on the contrary, a slight decrease in activity which was also observed in the frog just before the Na^{24} efflux peak Though the possibilities of a squeezing effect in this cyclic efflux cannot be disregarded (such an effect is suspected in the case of frog cardiac tissue, and is probably very weak in mammalians), this difference of behaviour between these two species might be interpreted as a difference in the pattern of potassium permeability during the electrical response in the rat and guinea pig The slight decrease in Na^{24} leakage occurring at the very beginning of the action potential (preceding the mechanical systolic activity) and visible in mammalians as well as in amphibians could reflect a sudden influx of Na into the cells in connection with the depolarization in the *electrically stimulated* preparations In the case of the *spontaneously beating* preparations the Na influx could precede the depolarization The presented results must be discussed critically in view of possible artifact producing mechanisms as well as in the light of experimental findings on oscillating model systems described by Teorell (1968)

This study was supported by contract DGRST Paris France and the Swedish Medical Research Council (grant B68 14X 621 02)

References

Brady A J and J M Woodbury The sodium potassium hypothesis as the basis of electrical activity in frog ventricle *J Physiol* (Lond) 1960 *154* 385—407

Coraboeuf E C Kayser and Y M Gargouil La repolarisation du myocarde au cours de l hypothermie chez trois especes de Mammiferes Cobaye Spermophile et Rat blanc *C R Acad Sci* (Paris) 1956 *243* 1673—1676

Coraboeuf E and G Vassort Effets de la Tetrodotoxine du tetraethylammonium et du manganese sur l activite du myocarde de Rat et de Cobaye *C R Acad Sci* (Paris) 1967 264 D 1072

Coraboeuf E F Zacouto Y M Gargouil and J Laplaud Mesure de la résistance membranaire du myocarde ventriculaire de Mammiferes au cours de l activite *C R Acad Sci* (Paris) 1958 *246* 2934—2937

Delahayes J and E Coraboeuf Fuite de potassium au cours du cycle cardiaque chez le Mammifere *C R Soc Biol* (Paris 1967 *161* 519

Danielson B J Delahayes and L Sjostrand Technical and practical aspects of spectrometry for determination of radioactive isotopes in mixtures *Acta Soc Med Upsal* 1966 *71* 173—196

Danielson B K J Öbrink and L Sjostrand Efflux of sodium and potassium from isolated frog heart during the cardiac cycle *Acta Soc Med Upsal* 1962 *67* 179—198

HODGKIN, A L and A F HUXLEY, Currents carried by sodium and potassium ions through the membrane of the giant axon of Loligo *J. Physiol* (Lond) 1952 *116* 449—472
HUTTER, O F and D NOBLE, Rectifying properties of Cardiac Muscle *Nature* (Lond) 1960 *188* 495
LAMB, J F and J A S MCGUIGAN, Potassium efflux from frog ventricle during the action potential *J Physiol* (Lond) 1966 *185* 32P
LORBER, V, J L WALKER, E A GREENE, M H MINARER and M J PAK, Phasic efflux of potassium from frog ventricle *Amer J Physiol* 1962 *203* 253—257
NOBLE, D, A modification of the Hodgkin-Huxley equations applicable to Purkinje fiber action and pacemaker potential *J. Physiol* (Lond) 1962 *160* 317—352
OFFNER K J and H R ULFENDAHL Gammaspectrometry for analysis of mixtures of radioisotopes [illegible] *J appl rad Isotopes* 1959 *5* 99—105
PAK, M J [illegible] C K. LOH and V LORBER, Measurement of tracer [illegible] *Physiol* 1966 *211* 1455—1460
REVIAL, F [illegible] ARNIER and Y M GARGOUIL, Application de la technique de perfusion à débit constant à l'etude des flux de ^{42}K au niveau du cœur isolé de rat *C R Soc Biol* (Paris) 1965 *159* 2009
SCHREIBER, S S, Potassium and sodium exchange in working frog heart Effects of overwork on external concentration of potassium and ouabain *Amer J Physiol* 1956 *185* 337—347
SJÖSTRAND, U, Analysis of ionic tracer movements during single heart cycles *Acta physiol scand* 1964 *61* Suppl 227
TEORELL, T, Electrokinetic membrane processes in relation to properties of excitable tissues I Experiments on oscillatory transport phenomena in artificial membranes *J gen Physiol* 1959 *42* 831—845
TEORELL T Parametric ionic pumping in a metastable wet artificial membrane system In press 1968
WEIDMANN S *Elektrophysiologie der Herzmuskelfaser*, H Huber, Bern 1956
WILDE W S and J M O'BRIEN The time relation between potassium (^{42}K) outflux action potential and the contraction phase as revealed by the effluogram *Abstr 19th int Physiol Congr* 1953 889—890

Acta physiol. scand. 1969. 76. 49—57

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Embryological Observations of the Ductus Arteriosus in the Guinea-Pig, Rabbit, Rat and Mouse. Studies on Closure of the Ductus Arteriosus. IV

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Received 29 July 1968

Abstract

HORNBLAD, P. Y. *Embryological observations of the ductus arteriosus in the guinea-pig, rabbit, rat and mouse. Studies on closure of the ductus arteriosus IV.* Acta physiol. scand. 1969. *76.* 49—57.

Fetuses of guinea pigs, rabbits, rats and mice were obtained by caesarean section in late pregnancy. They were immediately sacrificed, and fixed by whole body freezing, after which transverse cryostat sections of the ductus arteriosus were prepared for morphological observations. The inner diameter and wall thickness of the ductus were measured at its narrowest part. The descending thoracic aorta of full term rat fetuses was treated in the same way as the ductus.

In all species, the mean inner diameter of the ductus increased with fetal age, and was accompanied in the guinea pig and rabbit by a gain in thickness of the media. The development of an intima was most evident in rabbit fetuses. In all species, the structure of the ductal wall was comparable in the youngest and oldest fetuses. [illegible]

Material obtained at caesarean section has been used in a great number of morphological, physiological and pharmacological investigations on closure of the ductus arteriosus reviewed by Moss, Emmanouilides and Duffie (1963). The properties of the prenatal ductus might, however, differ from those of the ductus in the newborn. A delayed ductal closure after induced labour has, in fact, been reported in the guinea-pig (Record and McKeown 1955b). This might be explained by a certain degree of immaturity, since the ductus has been considered not to be morphologically mature until birth. An intimal proliferation (see Sciacca and Condorelli 1960 for references) or a dissociation of the inner media (Meyer and Simon 1960, Hoffmann 1964) has been described as a prenatal preparation for closure of the ductus

The whole body freezing technique permits instantaneous, permanent fixation of the ductus arteriosus (Hörnblad and Larsson 1967a) This technique was applied in studies of the prenatal ductus with respect to general growth, development of the intima and presence of structural changes in the media Four different species were studied, since species differences have been shown to exist in the normal closure rate (Hörnblad 1967)

The main purpose of this study was thus to investigate whether morphological differences are present between the prenatal and newborn ductus arteriosus If such differences do exist, physiological and pharmacological experiments on ductal closure would not be justified on fetuses obtained at caesarean section

Material and methods

Guinea pig Multiparous pregnant guinea pigs obtained from a commercial supplier were kept as described elsewhere (Hornblad 1967) For mating one male was placed with the newly delivered female for 2 days Caesarean section was performed under ether anesthesia on 3
it The age of the fetuses used
(Table I) Gestation period 68

mating were obtained from a
laboratory, as described in an
under ether anesthesia Fetuses
25 and 30 days old from 4 litters were used for this study (Table I) A number of the 25-day old fetuses were not included in the present material as their central vessels including the ductus were more or less collapsed. Gestation period 31 days (Handbook of Biological Data 1956

Rat Virgin rats of Sprague Dawley strain obtained from a commercial supplier were kept under conditions described in earlier investigations (Hornblad and Larsson 1967a) They were
sperms in the vaginal smear on
station Totally 12 animals were
19th 20th or 21st gestation day
l Data 1956)
were used (Larsson 1960 1962
Hornblad 1967) They were mated overnight and the day on which vaginal plug was observed was denoted as zero day of gestation Totally 10 animals were surgically delivered under ether anesthesia on the 17th, 18th 19th or 20th gestation day (Table I) Gestation period 20 days (Handbook of Biological Data 1956)

The fetuses were carefully removed from the uterus and sacrificed and fixed by whole body freezing (Hornblad and Larsson 1967a) Only fetuses which showed spontaneous body movements and were extracted in intact membranes were frozen The offspring of the guinea pig and rabbit were immersed in liquid nitrogen and those of the rat and mouse in isopentane cooled by dry ice (Hornblad 1967)

After storage of the fetuses for a few days at $-18°$ C tissue blocks of the ductus arteriosus were trimmed and transversely sectioned at 10 μ in a cryostat In the guinea pig and rabbit every 20th section was saved In rats of 19 20 and 21 gestation days every 15th section was preserved and in those of 17 and 18 days every 10th section In the mouse of 19 and 20
was prepared from every 10th section in those
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pendicular plane The arithmetic means of these data were used for calculations of the mean ductal inner diameter and wall thickness in the different species (Hornblad and Larsson 1967a) The mean inner diameter and wall thickness of the middle thoracic aorta of the full term rats were obtained in the same way

TABLE I Distribution of the material and dimensions of the ductal inner diameter and wall thickness in fetuses of the different species and ages

Species	Gestation age (days)	Number of litters	Number of animals	Mean inner diameter of ductus (μ)	Mean wall thickness of ductus (μ)
Guinea pig	48—50	3	3	1260	80
	61—63	2	4	2000	150
Rabbit	25	2	10	1100	80
	30	2	11	1600	100
Rat	17	2	7	405	40
	18	2	9	415	40
	19	2	11	515	35
	20	3	10	615	35
	21	3	16	690	40
Mouse	17	2	10	435	20
	18	2	8	470	20
	19	2	7	560	20
	20	2	8	520	20

Results

The inner diameter of the ductus arteriosus increased with progress of pregnancy in all four species (Table I) In the guinea pig and the rabbit, this was accompanied by an increase in wall thickness, mainly of the media (Table I, Figs 1*b* and *d*, 2*b* and *d*) The ratio $\frac{\text{wall thickness}}{\text{inner diameter}}$ in these two species had the same value in both age groups i e, 0 06—0 07, indicating the same relative wall thickness In the rat and mouse *on the other hand, no change in wall thickness could be demonstrated* (*Table I*, Figs 3*b* and *d*, 4*b* and *d*) In the rat this caused a decrease in the ratio $\frac{\text{wall thickness}}{\text{inner diamet}}$ from 0 10 in the 17-day-old fetuses to 0 06 in the 21 day-old fetuses In the mouse, the ratio decreased only from 0 05 to 0 04 between the 17 and 20 day old fetuses, due to the small increase in the lumen during this period (Table I)

The ductus and other central vessels of all specimens used in this study showed as in earlier investigations a distension (Figs 1*a*, 2*a* 3*a* and 4*a*) which can be considered as physiological (Hornblad and Larsson 1967a 1967b Hornblad 1967) The straight elastic lamellas separated sheets of slender muscle cells in the media The endothelium lining the smooth inner surface usually had widely separated and flat nuclei The subendothelial zone was fairly narrow in the guinea pig, rat and mouse fetuses in all age groups (Figs 1*b* and *d* 3*b* and *d* 4*b* and *d*) In the rabbit, the 25-

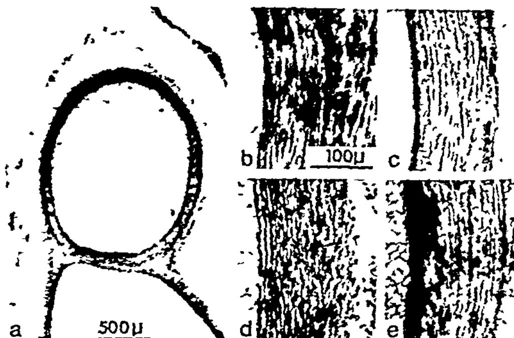


Fig. 1. Ductus arteriosus, fixed by whole-body freezing of guinea-pig fetuses delivered by caesarean section at the age of (a—c) 48—50 days (d and e), 61—63 days. a, b and d are photomicrographs of cryostat sections stained in hematoxylin and eosin, c and e of sections stained in Weigert's resorcin-fuchsin. b—e are printed in the magnification given in b.
The ductus of the 48—50-day-old fetus shows a round lumen (a) and thin walls (b). The ductal wall of the full-term fetus is thicker (d) and has a greater number of elastic lamellas than the younger fetus (c).

day-old fetuses showed a definite intima. In the 30-day-old fetuses fibroblasts and elastic and collagen fibres were more prominent (Fig. 2b—e).

The gain in ductal wall thickness in the guinea pig was accompanied by a corresponding increase in number of elastic lamellas from 15 to 20 approximately (Fig. 1c and e). Similarly the youngest rabbit fetuses had 16—17 elastic lamellas and the oldest approximately 20 (Fig. 2c and e). On the other hand, in the rat and mouse—which showed no increase in wall thickness—the number of lamellas, approximately 10 and 7 respectively, was the same in the youngest and oldest specimens (Figs. 3c, 4c and e). In all species the elastic lamellas of the ductus appeared more distinct in the oldest fetuses.

In 21-day-old rat fetuses the middle portion of the thoracic aorta had a mean inner diameter of 860 μ, which was 25 per cent more than that of the ductus at the corresponding age. Based on these figures the ductus has 64 per cent of the cross-sectional area of the descending aorta in the rat at full term. The wall thickness of the aorta was only 30 μ, which was 25 per cent less than that of the ductus (Fig. 3c and e). The ratios $\frac{\text{wall thickness}}{\text{inner diameter}}$ of the aorta and the ductus therefore differed considerably, being 0.03 and 0.06, respectively.

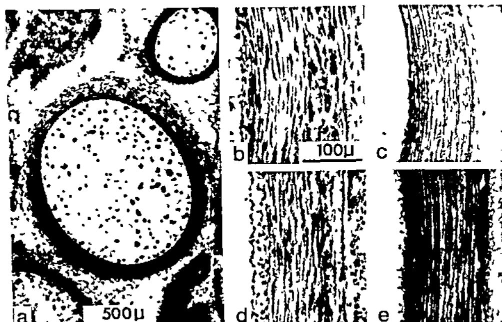


Fig 2 Ductus arteriosus fixed by whole-body freezing, of rabbit fetuses, delivered by caesarean section at the age of *a—c*, 25 days, *d* and *e*, 30 days *a* *b* and *d* are photomicrographs of cryostat sections stained in hematoxylin and eosin *c* and *e* of sections stained in Weigert's

(b) than both ages

Discussion

The demonstrated increase in lumen width of the ductus arteriosus indicates progressive enlargement during late pregnancy The increase in the ductal wall thickness in the guinea-pig and rabbit was due mainly to an increase in the media The development of the intima was most evident in the rabbit

An increase in the ductal lumen during pregnancy was reported in the human being by Piorier and Nicolas (1912) and Bakker (1960) However a decrease in its lumen during the last part of gestation in man and immediately before birth in the rat was described by van Ingen (1964) In an investigation by Ode (1952), the lumen of the human ductus was found to be almost constant during the second half of pregnancy whereas in the rat a marked decrease was recorded during this period In the calf no increase in the lumen could be demonstrated during the last two months of gestation (Harms 1966) The results of the present investigation showed in all species an increase in the ductal lumen during the last part of gestation In full term fetuses of the rat and the mouse, the inner diameter was almost the same as that observed in newborns (Hornblad 1967) In the guinea-pig on the other hand, the fetuses had a wider lumen than the newborns, probably due to variations in size of the animals (Hornblad 1967) Similarly, the full term fetuses of the rabbit showed a larger ductal lumen than the newborns (Hörnblad 1967) The two groups cannot

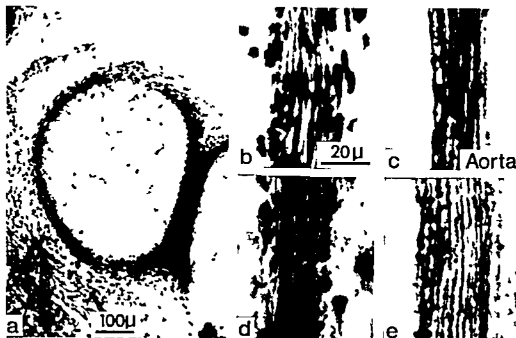


Fig. 3. Ductus arteriosus and aorta fixed by whole body freezing of rat fetuses delivered by caesarean section at the age of *a* and *b* 17 days, *c*—*e* 21 days. *a*, *b* and *d* are photomicrographs of cryostat sections stained in hematoxylin and eosin, *c* and *e* of sections stained in Weigert's resorcin fuchsin. *b*—*e* are printed in the magnification given in *b*.
The ductus of the 17 day-old fetus (*a* and *b*) shows a round lumen and the same wall thickness as that of the full term fetus (*d*) with narrow intimal layers. Greater stainability for elastic fibres is found in the aorta (*c*) than in the ductus (*e*), both vessels showing straight elastic lamellas.

however be compared, as some ductuses of the newborns had started to close when they were fixed (Hornblad 1967). *It is interesting to note that the ratio* $\frac{\text{wall thickness}}{\text{inner diameter}}$ was the same in full term fetuses of the guinea pig, rabbit and rat as in the newborns of the pig, guinea pig and rat (Hornblad 1967).

Ode (1952) reported an increase in ductal wall thickness in the human being and the rat, most marked during the last half of gestation. Calculations of the ratio $\frac{\text{wall thickness}}{\text{inner diameter}}$ from Ode's data on full term specimens give a value of 0.7, which is about 10 times that obtained in the present investigation. High values of this ratio are also obtained from data or illustrations given in investigations in man (Gräper 1921, Swensson 1939), calf (Harms 1966), pig (Schaeffer 1914), guinea pig (Record and McKeown 1953a, Sciacca and Condorelli 1960), rabbit (Sorcetti 1952) and rat (van Ingen 1964). The illustrations in the aforementioned papers support our view (Hornblad and Larsson 1967a) that a postmortem contraction of the ductus caused the thickening of the wall, as suggested earlier by Danesino, Reynolds and Rehman (1955), Record and McKeown (1953a) and Meyer and Simon (1960).

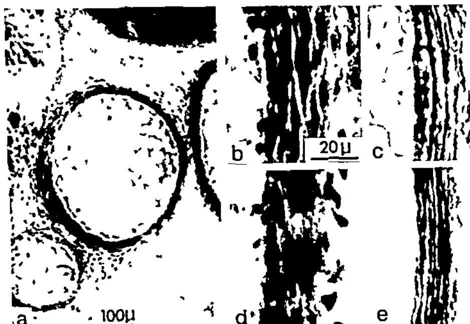


Fig 4 Ductus arteriosus fixed by whole body freezing of mouse fetuses delivered by caesarean section at the age of *a—c* 17 days *d* and *e* 20 days *a b* and *d* are photomicrographs of sections stained in hematoxylin and eosin *c* and *e* of sections stained in Weigert s resorcin fuchsin *b—e* ...

The ... nd lumen *(a)* Its wall thickness *(b)* is the same ... h ages only a narrow intimal layer can be observ ... stinct staining of the elastic fibres than the younger fetus *(c)* although the number of elastic lamellas is not increased

The increase in wall thickness has been regarded by other authors as the normal development of the ductus caused mainly by proliferation of intimal cells (Variot and Cailliau 1920 Graper 1921 Mělka 1926 Swensson 1939 Horwath and Szabo 1940 Jager and Wollenman 1942 Ode 1952 Patten 1953 Sciacca and Condorelli 1960 Hoffmann 1964 van Ingen 1964) The proliferation and a simultaneous increase in mucoid substances has been stated to cause an antenatal closure in the guinea pig (Sciacca and Condorelli 1960) Most of the authors cited above did however interpret the antenatal intimal growth as a preparatory step in postnatal closure Swensson (1939) and Bakker (1960) applied hydrodynamic aspects to the proliferations The present investigation on the guinea pig rabbit rat and mouse does not give support to the concept of an antenatal intimal proliferation of importance for closure of the ductus A proliferation of the intima does not even postnatally contribute to ductal closure in the initial phase (Hornblad and Larsson 1967b Hornblad 1967) Moreover no dissociation of the inner media was observed which has been stated to take place in man during the last part of gestation (Meyer and Simon 1960 Hoffmann 1964)

It was previously demonstrated that earlier used morphological techniques cause considerable postmortem contraction of both ductus arteriosus and aorta (Hornblad

and Larsson 1967a) The effect on the ductus is however, greater than that on the aorta The media of the ductus has therefore usually been described as substantially looser than that of the aorta, and with a thickness about twice that of the aorta (Graper 1921, Swensson 1939, Jager and Wollenman 1942, Ode 1952 Danesino Reynolds and Rehman 1955, Holmes 1958 van Ingen 1964) This is in contrast to the present finding of a ductal wall thicker than the aorta by only one third and exhibiting closely packed lamellas Furthermore, it can be calculated from the data given by Ode (1952) that the cross sectional area of the ductus in the full term rat fetuses is only 15 per cent of that of the descending aorta and in the human as low as 6 per cent The great discrepancy between Ode s figure of 15 per cent and the present one of 64 per cent in rat fetuses can be explained by the postmortem contraction of the ductus

Results of measurement of the areas of the ductus and the aorta support the view that the ductus contributes much of the blood to the descending aorta (Lind 1964) Moreover, it is close to a report by Patten and Toulmin (1930), quoted by Smith (1959) that the ductus at term has nearly half the cross sectional area of the descending aorta On the other hand, Bakker (1960) suggested approximately the same calibre of the ductus arteriosus and the aorta on the basis of studies of fetal human autopsy material However his technique of expanding the vessels during fixation might have caused an overdistension of the ductus, as described for other muscular arteries by Meyer (1958) It is interesting to note that the ratio $\frac{\text{wall thickness}}{\text{inner diameter}}$ of the rat thoracic aorta was found to be the same as that reported by Citters Wagner and Rushmer (1962) in small dog mesenteric arteries, quick frozen with the intraluminal pressure maintained

In the present investigation it was demonstrated that the ductus of the full term fetus has morphological similarities to that of the newborn animal It therefore seems justified to use fetuses obtained by caesarean section late in pregnancy for studies of the ductal closure mechanism even if it cannot be excluded on the basis of the present study that physiological immaturity of the ductus may exist in the full term fetus

r K Sune Larsson Department of Anatomy and or his critical reading of this manuscript and for f support has been received from the Swedish 14P 993 03) and from the Association for the Aid

References

BAKKER P M *Morfogenese en Involutie van de Ductus Arteriosus bij de Mens* Thesis Haag Mouton and Co 1962
CITTERS R L VAN B M WAGNER and R F RUSHMER Architecture of small arteries during vasoconstriction *Circulat Res* 1962 *10* 668—675
CONN H J M A DARROW and V M EMMEL *Staining Procedures* Baltimore Williams and Wilkins Co 2 ed 1960
DANESINO V L S R M REYNOLDS and I H REHMAN Comparative histological structure of the human ductus arteriosus according to topography age and degree of constriction *Anat Rec* 1955 *121* 801—831

z nach der Geburt III Ductus Botalli Z

Philadelphia and London, W B Saunders

Harms D, Über den Bau und Verschluss des Ductus arteriosus Botalli des Rindes Z Zellforsch 1966 72 344—363

Hoffmann, E, Die Obliteration des Ductus arteriosus Botalli *Langenbecks Arch klin Chir* 1964 *306* 289—314

Holmes, R. L., Some features of the ductus arteriosus *J Anat* (Lond) *92* 304—309

Hörnblad, P Y, Studies on closure of the ductus arteriosus III Species differences in closure rate and morphology *Cardiologia* 1967 *51* 262—282

Hörnblad, P Y and K S Larsson, Studies on closure of the ductus arteriosus I Whole-body freezing as improvement of fixation procedures *Cardiologia* 1967a *51* 231—241

Hörnblad P Y and K S Larsson, Studies on closure of the ductus arteriosus II Closure rate in the rat and its relation to environmental temperature *Cardiologia* 1967b *51* 242—252

Horvath, L and J Szabó, Beiträge zum Problem der Struktur und des Verschlusses des Ductus Botalli *Dtsch Z ges gerichtl Med* 1940 *33* 175—184

Ingen, H C van, *Morfologische Aspecten van het Sluitingsproces van de Ductus Arteriosus* Thesis Leiden, Drukkerij "Luctor et Emergo" 1964

Jager B V and O J Wollenman, An anatomical study of the closure of the ductus arteriosus *Amer J Path* 1942 *18* 595—613

Larsson, K S, Studies on the closure of the secondary palate II Occurrence of sulphomucopolysaccharides in the palatine processes of the normal mouse embryo *Exp Cell Res* 1960 *21* 498—503

Larsson K S, Studies on the closure of the secondary palate III Autoradiographic and histochemical studies in the normal mouse embryo *Acta morph neerl scand* 1962 *4* 349—367

Lind J L Stern and C Wegelius, The fetal circulation In *Human Fetal and Neonatal* ... Thomas, Publisher 1964 pp 6—27

... und Obliteration des Ductus arteriosus

... von Arterien und Venen *Verh dtsch*

Meyer, W W and E Simon, Die präparatorische Angiomalacie des Ductus arteriosus Botalli als Voraussetzung seiner Engstellung und als Vorbild krankhafter Arterienveränderungen *Virchows Arch path Anat* 1960 *333* 119—136

Moss, A J, G Emmanouilides and E R Duffie, Closure of the ductus arteriosus in the newborn infant *Pediatrics* 1963 *32* 25—30

Ode E, *De Ductus Arteriosus* Thesis Leiden, A W Sijthoff's Uitgeversmaatschappij N V 1951

Patten B M, Postnatal circulatory changes In *Human Embryology* New York and Toronto, The Blakiston Co Inc 2 ed 1953 pp 696—705

Patten B M and K Toulmin, Certain measurements of the fetal heart and their significance *Anat Rec* 1930 *45* 235

Poirier P and A Nicolas, Description des artères Système de l'artère pulmonaire In *Traité D Anatomie Humaine* (A Nicolas, ed) Paris, Masson et C^e, Editeurs, 3 ed 1912 pp 201—203

Record R G and T McKeown, Anatomical closure of the ductus arteriosus in the guinea pig *Clin Sci* 1955a *14* 213—223

Record R G and T McKeown The effect of reduced atmospheric pressure on closure of the ductus arteriosus in the guinea pig *Clin Sci* 1955b *14* 225—233

Schaeffer J P, The behavior of elastic tissue in the postfetal occlusion and obliteration of the ductus arteriosus (Botalli) in Sus Scrofa *J exp Med* 1914 *19* 129—143

Sciacca A and M Condorelli Involution of the ductus arteriosus A morphological and experimental study, with a critical review of the literature *Bibl cardiol* (Basel 1960 Fasc 10

Smith C A The circulatory system In *The Physiology of the Newborn Infant* Springfield, Illinois Charles C Thomas Publisher 3 ed 1959 pp 102—152

Sorcetti F Analogie di struttura e di meccanismo di obliterazione tra canale arterioso ed arterie ombelicali nei mammiferi *Riv Biol* 1952 *44* 93—116

Swensson Å Beitrag zur Kenntnis von dem histologischen Bau und dem postembryonalen Verschluss des Ductus arteriosus Botalli *Z mikr anat Forsch* 1939 *46* 275—298

Variot G and F Cailliau Recherches sur le processus d obliteration du canal arteriel *Soc Med Hop Paris* 1920 *44* 1598—1604

Acta physiol. scand. 1969. 76. 58—66

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Effect of Oxygen and Umbilical Cord Clamping on Closure of the Ductus Arteriosus in the Guinea-Pig and the Rat. Studies on Closure of the Ductus Arteriosus. VI

By

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Received 29 July 1968

Abstract

HORNBLAD P Y *Effect of oxygen and umbilical cord clamping on closure of the ductus arteriosus in the guinea-pig and the rat Studies on closure of the ductus arteriosus VI* Acta physiol scand 1969 76 58—66

ll term guinea pigs were delivered by caesarean section Living fetuses with intact or interrupted umbilical circulation were allowed to breathe, for periods of 5 min, air or nitrogen or air followed by nitrogen Animals sacrificed immediately on birth served as controls Sacrifice was performed in liquid nitrogen —Spontaneously delivered rats were either sacrificed immediately on birth or kept up to 4 hrs at 37° C in air or 8 % O_2 in N_2 before sacrifice in isopentane cooled by dry ice In addition the heart rate of newborn rats, kept at the same temperature and in the same atmospheres was recorded — Changes in ductal inner diameter and wall thickness were measured in cryostat sections of both species Oxygen caused marked constriction of the ductus in the guinea pig Further constriction was found in animals with a clamped umbilical cord No evidence was obtained of an effect of nitrogen inhalation neither on the open nor on the constricted ductus In the rat reduced atmospheric oxygen concentration caused a delay in ductal closure rate Lowering of the heart rate was also observed The present results—obtained *in vivo* in the guinea pig and rat—show that oxygen is essential for closure of the ductus arteriosus independent of the rate of its closure

Oxygen is the most extensively studied factor involved in closure of the ductus arteriosus Its constrictive effect is now generally accepted although its mechanism of action is still unknown However, narrowing of the ductus has also been observed during asphyxia (Born *et al* 1956) In experiments with maximal reduction of the oxygen concentration nitrogen has been stated to cause dilatation of the constricted ductus (Kennedy and Clark 1941 1942 Born *et al* 1956 Reis and Anderson 1964 Clamping of the umbilical cord is another factor which has been reported to cause constriction of the ductus arteriosus (Ardran *et al* 1952) Closure of the ductus has been observed even before clamping of the umbilical cord and onset of ventilation (Barclay *et al* 1938)

The conflicting results may be due mainly to the use of different techniques and

different species It therefore seems justified to reinvestigate some of these factors, using a simple morphological technique The whole body freezing technique has been shown to be suitable for registering the rapid changes in the ductal closure process (Hörnblad and Larsson 1967a Hornblad 1967) Such studies should, moreover, be performed on animals showing different closure rates, such as the guinea pig and rat (Hornblad 1967, 1969)

Material and methods

Guinea pig

Multiparous pregnant guinea pigs with unknown date of mating were obtained from a commercial supplier They were brought to the laboratory when distinct symphysiolysis was diagnosed Spontaneous delivery was then approaching (Record and McKeown 1955a), and most animals were therefore delivered by caesarean section under ether anesthesia on the same day The others were kept for a few days in the laboratory under conditions described earlier (Hornblad 1967), while awaiting adequate symphysiolysis At caesarean section the lower abdominal wall was opened in the midline after infiltration with 2—4 ml of a 0 5 % local anesthetic[1] The lower part of the animal was then immersed in saline kept at 38° C (Hand book of Biological Data 1956) A portion of the uterus was carefully pulled out and a fetus extracted below the surface of the saline, through an incision opposite the attachment of the placenta One fetus was treated and sacrificed before the next was extracted The animals were divided into different experimental groups with respect to birth order within the litters

Two sets of experiments were performed A The effect of clamping of the umbilical cord and spontaneous ventilation with air or nitrogen on the initial ductal closure was studied in 27 animals from 10 litters (Experiment set A Table I) B The effect of nitrogen inhalation after an initial period of spontaneous ventilation with air on the ductal inner diameter was studied in 15 animals from 5 litters (Experiment set B Table I)

Clamping was achieved by applying a hemostat to the cord when the membranes were broken and the head of the fetus was raised above the surface of the saline (A2 and A4 B1—B3) The fetuses were allowed to breathe air (A1 and A2 B1), nitrogen (A3 and A4) or first air and then nitrogen (B3) for a period of 5 min, or air for 10 min (B2) Attempts were made to induce spontaneous respiration by pinching the skin and inflating the lungs The head of fetuses intended to breathe nitrogen was raised into an inverted glass funnel [illegible] and continuously supplied with nitrogen [illegible] supervised with respect to palpable heart [illegible]ients and reaction to pain The pulsations [illegible]mping and during the experiments when unclamped.

The experiment started at the moment when the membranes were broken and the head raised above the saline After 5 min (A1—A4 B1) or 10 min (B2 and B3), the cord was cut and living fetuses were sacrificed and fixed by immersion in liquid nitrogen One group of fetuses was sacrificed immediately on removal from the uterus (A5)

Rat

Primiparous rats of Sprague Dawley strain obtained from a commercial supplier, were kept under conditions described in our earlier papers (Hornblad and Larsson 1967a) On the 21st and 22nd days after mating the animals were continuously observed for beginning of labour

Totally 92 newborns from 11 litters were used in this study One group of newborn animals was sacrificed immediately on delivery All others were left for 2 min with the mother allowing her to remove the fetal membranes and placenta According to birth order these newborns were then placed alternately in one of two sets of special glass containers for incubation at 37° C (Hornblad and Larsson 1967b) Each contained in one set was flushed at 0 5 l/min with air and in the other with a gas mixture of 8 ± 1 % oxygen in nitrogen[2]

The animals were kept in the containers to the age of 30 min 1 2 3 or 4 hrs and then sacrificed by immersion in isopentane cooled by dry ice

In both species transverse cryostat sections were prepared and measurements made of the inner and outer diameter of the ductus at its narrowest part as described earlier Hornblad

[1] Citanest® Astra Sodertalje Sweden
[2] Obtained from AGA AB Lidingo Sweden

TABLE I Dimensions of inner diameter and wall thickness of the ductus arteriosus of guinea pig fetuses with an intact or clamped umbilical cord after administration of air or nitrogen

Treatment: Umbilical circulation	Treatment: Atmosphere administered	Age in minutes	Experimental group	Number of litters	Number of animals	Inner diameter of ductus (μ) Mean	Inner diameter of ductus (μ) SD[1]	Wall thickness of ductus (μ) Mean	Wall thickness of ductus (μ) SD
Intact	Air	5	A1	6	7	920	700	250	120
Interrupted	Air	5	A2	6	6	250	110	360	40
Intact	Nitrogen	5	A3	6	6	1750	440	140	70
Interrupted	Nitrogen	5	A4	4	4	1910	360	100	10
0 minute control		0	A5	4	4	2030	760	130	20
Interrupted	Air	5	B1	5	5	410	260	350	80
Interrupted	Air	10	B2	5	5	310	160	380	90
Interrupted	Air and Nitrogen	5+5	B3	4	5	680	600	340	130

[1] SD Standard deviation (Ostle 1954)

and Larsson 1967a. Based on these data the mean ductal inner diameter and wall thickness in the different experimental groups were calculated (Tables I and II). A one tailed significance analysis with Student's *t* test was performed (Ostle 1954). For morphological studies selected sections from the middle portion of the ductus in the rat were stained for elastic and collagen fibres (Conn, Darrow and Emmel 1960).

In a separate experiment the influence of reduced oxygen supply on the heart rate in the rat was investigated. The recordings were made on 36 newborns from 3 litters (Table III) during 4 hrs after delivery as described in a previous communication (Hornblad and Larsson 1967b).

Results

Effect of spontaneous breathing of air or nitrogen and of umbilical cord clamping on ductal closure in the guinea pig

The inner diameter of the ductus arteriosus 5 min after surgical delivery was markedly reduced in animals allowed to breathe air (A1 and A2, Table I). When the circulation through the umbilical cord was maintained intact (A1), the mean inner diameter was decreased to 45 per cent of the 0 minute mean (A5). In animals with a clamped umbilical cord (A2) it was reduced to 12 per cent. Animals kept in an atmosphere of pure nitrogen for 5 min both with an intact (A3) [illegible] clamped (A4) umbilical cord, showed an open ductus [illegible] I) [illegible] the air breathing animals with an intact plac[illegible] when the cord was clamped (A2). The m[illegible] and clamped

(A4) umbilical cord had values of 0 08 and 0 05 respectively (Table I) The ratio $\frac{\text{wall thickness}}{\text{inner diameter}}$ for the 0 minute specimens (A5) was 0 06

In animals with a clamped umbilical cord the value of the mean ductal inner diameter (Table I) was 310 μ following 10 min of breathing air (B2), 410 μ after 5 min (B1), and 680 μ when followed by a 5 min period in nitrogen (B3) The difference between the three groups was not, however, significant

After clamping of the cord all animals initially showed regular and frequent breathing Animals kept in the nitrogen atmosphere did not however, maintain regular breathing throughout the 5 min period Moreover their heart rate decreased and they became hypotonic Animals with a clamped umbilical cord and allowed to breathe air for 5 min showed—when subjected to nitrogen—within 1 min a muscular excitation followed by severe general hypotonia On the other hand when the placental circulation was left intact regular breathing could not be initiated in any of the 6 animals kept in nitrogen nor in 4 of the 7 animals kept in air

Effect of reduced oxygen supply on the ductal closure rate in the rat

The newborn rats kept in the low oxygen atmosphere showed a significantly decreased closure rate of the ductus arteriosus during the period investigated (Table II) After 30 min the ductal inner diameter of these animals was reduced to 63 per cent of the 0 minute mean and at 1 hr to 39 per cent while in the air breathing group the corresponding reductions were 37 per cent and 12 per cent respectively At 2 hrs of age when the constriction in the control group was almost complete—reduction to less than 6 per cent—the animals supplied with a lowered oxygen concentration showed a decrease to 23 per cent of the 0 minute mean and a further decrease to 13 per cent at 3 hrs No additional decrease was observed at 4 hrs The ductus of the control animals 3 and 4 hrs old lacked a lumen

Except for a greater lumen no morphological differences were observed in the ductus of the hypoxic animals and the controls of the same age An increase in wall thickness was observed concomitantly with the decrease in inner diameter of the ductus

The low oxygen supply caused a significantly lowered heart rate in the newborn rat (Table III) The surviving animals in this group were all cyanotic, and showed slow body movements in comparison to those breathing air All animals kept in air survived Of the 20 animals kept for 4 hrs in the low oxygen atmosphere while recording the heart rate 6 died within 2 hrs 5 of which were from the same litter

Discussion

It has been possible with the whole body freezing technique to reveal morphologically the constrictive effect of oxygen on the ductus arteriosus in the guinea pig and the rat Nitrogen inhalation did not cause dilatation or constriction of the ductus in the guinea pig Clamping of the umbilical cord was found in the present investigation to produce additional constriction in animals breathing air

TABLE II Inner diameter of the ductus arteriosus of newborn rats, supplied with normal or reduced atmospheric oxygen concentration and statistical comparison between the different groups

Atmosphere administered	Age in minutes	Number of litters	Number of animals	Inner diameter of ductus (μ) Mean	SD[1]	Significance[2]
Control	0	10	14	880	60	
Normal[3]	30	7	7	325	210	x
Reduced[4]		8	9	555	330	
Normal	60	8	10	105	55	xx
Reduced		7	9	345	195	
Normal	120	2	7	50	30	xxx
Reduced		6	7	200	50	
Normal	180	6	6	0		xxx
Reduced		5	6	115	40	
Normal	240	8	9	0		xxx
Reduced		6	8	130	80	

[1] SD = Standard deviation (Ostle 1954)
[2] 0 = not significant x = almost significant, $p < 0.05$, xx = significant, $p < 0.01$, xxx = highly significant $p < 0.001$
[3] Normal = Air
[4] Reduced = 8 ± 1 % O_2 in N_2

In experimental studies on factors influencing ductal closure, it is of utmost importance to obtain the ductus instantaneously fixed at times desired Furthermore, for judging the degree of closure the parameter chosen must represent the true ductal lumen

The role of oxygen in closure of the ductus arteriosus has been investigated *in vitro* (Assali *et al* 1963, Kovalčík 1963) on isolated preparations (Born *et al* 1956, Reis and Anderson 1964) and in vivo (Kennedy and Clark 1941, 1942, Record and McKeown 1955a 1955b Born *et al* 1956, Wilcox Roberts and Carney 1962 Assali *et al* 1963) Exposure or delayed fixation of the ductus was, however necessary in these investigations Such treatment would presumably have caused a constriction of the ductus since this vessel contracts on mechanical stimulation and postmortem as demonstrated earlier (Hornblad and Larsson 1967a, Hornblad 1967)

Direct measurements of the inner diameter on cryostat sections from whole body frozen animals seem to be satisfactory for evaluation of the degree of ductal closure Measurements or direct observations of the outer diameter of the ductus do not appear to be reliable (Barclay *et al* 1938) The results of X-ray examinations of the

ductus after injection of radio-opaque media are difficult to interpret, since absence of contrast medium in the ductus does not provide conclusive evidence of a closed ductus (Prec and Cassels 1955, Condorelli and Ungari 1960) Moreover the injection itself might produce hemodynamic disturbances (Dawes, Mott and Widdicombe 1955) Information about the ductal lumen obtained from measurements of the blood flow through the ductus may be limited (Smith, Morris and Assali 1964) The dye dilution method has been reported to be highly sensitive in demonstrating changes in blood shunting through the ductus in man (Prec and Cassels 1955, Condorelli and Ungari 1960, Hanson *et al* 1967) In view of technical difficulties this method does not at present seem to be applicable to small experimental animals

The marked constriction of the ductus arteriosus demonstrated in the guinea pig after breathing air confirms the concept of a relation between oxygen and closure of the ductus (Kennedy and Clark 1942, Born *et al* 1956, James and Rowe 1957, Assali *et al* 1963, Kovalčik 1963, Moss *et al* 1964, Reis and Anderson 1964) The observed delay in closure rate in rats, kept in a low oxygen atmosphere, further supports this view It is interesting to note that oxygen is of crucial importance for closure of the ductus, independent of the ductal closure rate, which is high in the guinea pig compared to that in the rat, as demonstrated previously (Hornblad 1967) The fact that no constriction of the ductus was observed after inhalation of nitrogen supports the earlier concept that ductal closure is independent of mechanical lung inflation (Born *et al* 1956 Assali *et al* 1963)

On the other hand dilatation of the ductus has been described in experiments designed to decrease the blood or tissue oxygen tension by nitrogen. In *in vitro* experiments, sections of the ductus arteriosus from fetal lambs and guinea pigs were reported to dilate when bubbling of air through the organ bath was replaced by nitrogen (Kovalčik 1963) Similarly, dilatation of a partially closed ductus was observed in isolated preparations of the lamb ductus when the oxygen tension of the perfusing blood was lowered (Born *et al* 1956 Reis and Anderson 1964) In the guinea pig an increase in the ductal outer diameter was observed *in vivo* on administration of nitrogen (Kennedy and Clark 1942) In the present investigation, however the guinea pig fetuses with a clamped umbilical cord rapidly deteriorated when nitrogen was substituted for air In these animals, no significant dilatation of the ductus could be demonstrated A possible explanation of the dilatation reported by Kennedy and Clark (1942) is that the umbilical circulation was maintained intact

It has been reported that clamping of the umbilical cord alone was sufficient to produce closure of the ductus in fetal lambs (Ardran *et al* 1952) In an extensive study of fetal lambs constriction of the ductus was observed during asphyxia after tying of the umbilical cord (Born *et al* 1956) The authors interpreted the constriction as caused by increased catecholamine secretion from the adrenal medulla Assali and co-workers discussing this report postulated that the constriction might have been caused by a fall in cardiac output and pulmonary flow (Assali *et al*, Smith Morris and Assali 1964) In the present investigation, a constrictive

TABLE III Heart rate of newborn rats supplied with normal or reduced atmospheric oxygen groups

Atmosphere administered	Number of litters	Number of animals	Heart rate (cycles/min), Mean and SD[1]				
			15[2]	30	45	60	75
Normal[3]	3	16	258 40	322 22	338 14	339 14	338 17
Reduced[4]	3	14	231 41	271 21	290 11	291 20	293 18
Significance[5]			x	xxx	xxx	xxx	xxx

[1] SD = Standard Deviation (Ostle 1954)
[2] Initial recording 15 min after birth Subsequent recordings at 15 min intervals
[3] Normal = Air
[4] Reduced = 8±1 % O_2 in N_2
[5] See Table II

umbilical cord clamping was encountered only in the ductus of guinea pig fetuses allowed to breathe air Clamping of the umbilical cord might therefore be of secondary importance in producing a temporary condition of asphyxia which exaggerates the breathing The violent respiratory movements observed in the initial period of nitrogen inhalation following breathing air support this view This is in agreement with earlier reports of the stimulating effect on breathing of hypoxia (Cross Kullich and Hugget 1954) and of umbilical cord clamping (Harned *et al* 1961)

The lowering of the heart rate observed in newborn rats kept in an atmosphere of reduced oxygen is similar to observations on lambs *in utero* (Assali Holm and Sehgal 1962) Lowering of the heart rate in newborn rats also follows a decrease in the environmental temperature (Fairfield 1948) A decrease in the temperature from 37° C to 30° C reduced the heart rate from 340 to 210 cycles per minute without any effect on the ductal closure (Hornblad and Larsson 1967b) It is interesting to note that the moderate lowering of the heart rate from 330 to 300 cycles per minute, observed in the present investigation during hypoxia was accompanied by a significant decrease in closure rate These differences in the action of environmental factors on the ductal closure are still unknown

It is puzzling that the mechanism involved in ductal closure has not been disclosed although its relation to oxygen was demonstrated almost 30 years ago But many other factors are presumably involved Additional data seem therefore to be needed on the composition of the ductal wall and its physiology Our earlier studies in this field indicate that the whole body freezing technique combined with other procedures such as X ray, dye dilution and isotope techniques should be fruitful

concentration, during 4 hours after birth, and statistical comparison between the different

90	105	120	135	150	165	180	195	210	225	240
336	333	324	330	322	322	321	319	320	317	315
12	9	16	13	20	16	18	16	14	14	19
294	292	297	297	303	306	307	305	297	299	299
19	26	25	27	28	26	23	24	32	29	33
xxx	xxx	xxx	xxx	x	x	x	x	xx	x	x

I am greatly indebted to D
linska Institutet for his
supported by grants from
04) and from the Associat

References

ARDRAN G M G S DAWES M M L PRICHARD S R M REYNOLDS and D G WYATT, The effect of ventilation of the foetal lungs upon the pulmonary circulation *J Physiol* (Lond) 1952 *118* 12—22

ASSALI N S L W HOLM and N SEHGAL, Hemodynamic changes in fetal lamb in utero in response to asphyxia hypoxia and hypercapnia *Circulat Res* 1962 *11* 423—430

ASSALI N S J A MORRIS R W SMITH and W A MANSON Studies on ductus arteriosus circulation *Circulat Res* 1963 *13* 478—489

BARCLAY A E J BARCROFT D H BARRON and K J FRANKLIN X ray studies of the closing of the ductus arteriosus *Brit J Radiol* 1938 *11* 570—585

BORN G V R G S DAWES J C MOTT and B R RENNICK The constriction of the ductus arteriosus caused by oxygen and by asphyxia in newborn lambs *J Physiol* (Lond) 1956 *132* 304—342

CONDORELLI S and C UNGARI The period of functional closure of the foramen ovale and the ductus Botalli in the human newborn *Cardiologia* 1960 *36* 274—287

CROSS K W E M KILLICK and A ST G HUGGETT Hypoxia in fetal and neonatal life *Amer J Obstet Gynec* 1954 *68* 81—90

DAWES G S J C MOTT and J G WIDDICOMBE The patency of the ductus arteriosus in newborn lambs and its physiological consequences *J Physiol* (Lond) 1955 *128* 361—383

FAIRFIELD J Effects of cold on infant rats Body temperatures oxygen consumption electrocardiograms *Amer J Physiol* 1948 *155* 355—365

Handbook of Biological Data (W S Spector ed) Philadelphia and London W B Saunders Co 1956 p 343

HANSON J S R A ARCILLA W OH G WALLGREN and J LIND Quantitative studies of the

HORNBLAD P Y Embryological observations of the ductus arteriosus in the guinea pig rabbit rat and mouse Studies on closure of the ductus arteriosus IV *Acta physiol scand* 1969 *76* 49—57

HORNBLAD P Y and K S LARSSON Studies on closure of the ductus arteriosus I Whole body freezing as improvement of fixation procedures *Cardiologia* 1967a *51* 231—241

HORNBLAD P Y and K S LARSSON Studies on closure of the ductus arteriosus. II Closure rate in the rat and its relation to environmental temperature *Cardiologia* 1967b *51* 242—252

JAMES L. S and R D ROWE The pattern of response of pulmonary and systemic arterial pressures in newborn and older infants to short periods of hypoxia. *J Pediat* 1957 *51* 5—11

KENNEDY J A and S L CLARK Observations on the ductus arteriosus of the guinea pig in relation to its method of closure *Anat Rec* 1941 *79* 349—371

KENNEDY J A and S L CLARK Observations on the physiological reactions of the ductus arteriosus *Amer J Physiol* 1942 *136* 140—147

KOVALČIK V The response of the isolated ductus arteriosus to oxygen and anoxia *J Physiol* (Lond) 1963 *169* 185—197

MOSS A J, G C EMMANOUILIDES F H ADAMS and K CHUANG Response of ductus arteriosus and pulmonary and systemic arterial pressure to changes in oxygen environment in newborn infants. *Pediatrics* 1964 *33* 937—944

OSTLE B *Statistics in Research* Ames Iowa The Iowa State College Press 1954

PREC K J and D E CASSELS Dye dilution curves and cardiac output in newborn infants. *Circulation* 1955 *11* 789—798

RECORD R G and TH MCKEOWN The effect of reduced atmospheric pressure on closure of the ductus arteriosus in the guinea pig *Clin Sci* 1955a *14* 225—233

RECORD R G and TH MCKEOWN The effect of oxygen deficiency on closure of the ductus arteriosus in the newborn guinea pig *Clin Sci* 1955b *14* 711—714

REIS R L and R P ANDERSON Constriction of the ductus arteriosus Experimental observations in the newborn lamb *J surg Res* 1964 *4* 356—359

SMITH R W J A MORRIS and N S ASSALI Effects of chemical mediators on the pulmonary and ductus arteriosus circulation in the fetal lamb *Amer J Obstet Gynec* 1964 *89* 252—260

WILCOX B R W C ROBERTS and E K CARNEY The effect of reduced atmospheric oxygen concentration on closure of the ductus arteriosus in the dog *J surg Res* 1962 *2* 312—316

Acta physiol. scand. 1969. 76. 67—72

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The Mutual Influence of Gastrin and Secretin on the External Pancreatic Secretion in Dogs

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Received 1 August 1968

Abstract

HENRIKSEN, F W and H WORNING *The mutual influence of gastrin and secretin on the external pancreatic secretion in dogs* Acta physiol scand 1969 *76 67—72*

The external pancreatic secretion of fluid, bicarbonate and protein in 4 dogs with Thomas type fistulae has been studied in response to secretin (1 U/kg b w), gastrin (2 U/kg b w) and combined secretin—gastrin stimulation Gastrin exerted a stimulating effect on the pancreatic secretion of fluid, bicarbonate and protein and increased the bicarbonate concentration in the juice significantly above basal level The combined effect of secretin and gastrin on the secretion of fluid bicarbonate and protein did not differ from the summarized effect of the two agents given separately Potentation between secretin and gastrin was not demonstrated

Gregory and Tracy (1964 observed that the pure peptides gastrin I and II stimulated the pancreatic secretion, mainly of protein Preshaw, Cooke and Grossman (1965 a) made similar observations with a crude gastrin preparation and suggested the effect to be of physiological importance in the intact organism (Preshaw and Grossman 1965 b)

In contradistinction to the extensive studies of the interaction between nervous and hormonal stimulation of the pancreas, the mutual influence of different hormones has only been investigated to a limited extent Recently pancreozymin was reported to potentiate the pancreatic response to secretin (Brown *et al* 1967, Henriksen and Worning 1967) Preshaw (1966) who used combined stimulation with secretin and a crude gastrin preparation was unable to demonstrate any potentitation between these two hormones

In the present study the mutual influence of secretin and pure gastrin on the external pancreatic secretion in dogs has been investigated

Material and methods

Four mongrel dogs (b w 17 0—28 0 kg) were used After ligation of the accessory pancreatic duct a modified Thomas cannula (Thomas 1941) was inserted into the duodenum exactly opposite the main pancreatic duct The same type of cannula was inserted into the stomach at the most pendent part The dogs were used in experiments after a recovery period of at least 3 weeks

During the experiments the dogs were standing in a sling harness The gastric cannula was opened to prevent gastric secretion from entering the duodenum No gastric acid was observed in the duodenum in any of the experiments A glass catheter connected to a glass syringe by a short polyvinylchloride tube was inserted into the main pancreatic duct through the open duodenal cannula The pendent plunger of the syringe exerted a slight negative pressure The dead space of the collecting system was 0 4 ml

Except for small amounts of water the dogs were fasted for 18 hrs prior to the experiments The fasting pancreatic secretion was collected for at least 15 min After stimulation of the secretion the juice was collected in 10+10+20+20 min

The secretin preparation used was Secretin Boots (Boots Pure Drug Co Nottingham England) which contained about 25 Crick Harper and Raper units of pancreozymin per 100 units of secretin (information from the manufacturers) In the present work doses of secretin are expressed in Crick Harper and Raper units The gastrin (Leo Pharmaceutical Products Copenhagen Denmark) was administered in Leo units (1 Leo unit = 1 μg gastrin)

The entire dose response curve for [illegible]
secretion following stimulation with g [illegible]
gastrin 2 U/kg b w was determined [illegible]
injected into a peripheral vein in 10— [illegible]
sequent injections was at least 100 min on account of the refractory period previously demonstrated (Henriksen 1966) No more than three injections were given each day of experiments

The volume of the pancreatic secretion and the concentration of total CO_2 and of protein in the samples were measured The concentration of total CO_2 was determined according to Van Slyke's manometric method (1924) This concentration is referred to as bicarbonate The concentration of protein was estimated spectrophotometrically at 280 nm after appropriate dilution of the samples with Sørensen phosphate buffer pH 6 8 The extinction values were converted to concentrations of protein by a factor empirically determined through Kjeldahl's analysis on pooled canine pancreatic juice

The probability of difference was calculated by use of Student's t test in the individual dog and in the group of dogs according to the method described by Fisher (1958)

Results

The pancreatic secretion increased above nonstimulated level within 1 min after stimulation irrespective of the stimulant applied The response after secretin and after combined secretin and gastrin subsided to basal level within 30 min after gastrin it subsided within 10 min Consequently only the secretion in the first 10 min following stimulation was used in the calculations

Gastrin induced a significant increase in the bicarbonate concentration and in the secretion rates of fluid bicarbonate and protein in all dogs (Table I and II)

The mean secretion rates of fluid bicarbonate and protein after stimulation with secretin gastrin or both are illustrated in Table III—V for each dog as well as for the material as a whole

The secretion rates of fluid and bicarbonate after combined stimulation did not differ from the sum of the secretion rates after secretin and gastrin neither in the individual dog nor in the group of dogs (Table III—IV)

In one dog the secretion rate of protein after combined stimulation differed significantly from the summarized effect but calculated for the group of dogs no difference was observed (Table V)

TABLE I Pancreatic secretion rates of fluid (ml/min) and protein (mg/min) in the period 0—10 min after gastrin stimulation (2U/kg b w) as compared to the basal secretion rates

Dog no	Volume secretion rate		p**	Protein secretion rate		p**
	Basal	Gastrin		Basal	Gastrin	
64—208 m*	0 067	0 20	<0 001	2 8	11 7	<0 001
n	183	3		38	3	
65—231 m*	0 036	0 24	<0 001	2 3	18 3	<0 001
n	134	3		12	2	
65—271 m	0 065	0 20	<0 001	2 7	10 2	<0 001
n	97	3		18	3	
66—177 m	0 040	0 11	<0 001	1 6	4 7	<0 001
n	46	3		8	3	

*m mean
n number of observations
**p probability of difference

TABLE II Bicarbonate concentration (meq/l) and bicarbonate secretion rate (μeq/min) of the pancreatic juice in the period 0—10 min after gastrin stimulation (2U/kg b w) as compared to the basal levels

Dog no	Bicarbonate concentration			Bicarbonate secretion rate		
	Basal	Gastrin	p**	Basal	Gastrin	p**
64—208 m*	51 0	118 3	<0 001	4 7	24	<0 001
n	50	3		50	3	
65—231 m	61 3	105 0	0 01	3 7	26	<0 01
n	33	3		33	3	
65—271 m	68 6	123 7	0 001	6 9	25	< 0 001
n	35	3		35	3	
66—177 m	64 0	86 2	—	2 8	9	—
n	15	1		15	1	

*m mean
n number of observations
**p probability of difference

TABLE III Secretion rate of fluid (ml/min) in the period 0—10 min after stimulation with secretin (1 U/kg b w), gastrin (2 U/kg b w) or combined secretin gastrin

Dog no		Secretin	Gastrin	Combined	p**
64—208	m*	1 27	0 20	1 46	n s
	n	7	3	3	
65—231	m	1 31	0 24	1 50	n s
	n	9	3	3	
65—271	n	1 38	0 20	1 32	n s
	n	4	3	3	
66—177	m	1 34	0 11	1 59	n s
	n	8	3	3	
All	m	1 32	0 19	1 47	n s

m* mean
n number of observations
p** probability of difference between combined secretion rate and the sum of secretin and gastrin secretion rates

TABLE IV Secretion rate of bicarbonate (μeq/min) in the period 0—10 min after stimulation with secretin (1 U/kg b w) gastrin (2 U/kg b w) or combined secretin gastrin

Dog no		Secretin	Gastrin	Combined	p**
64—208	m*	162	24	200	n s
	n	7	3	3	
65—231	m	170	20	192	n s
	n	9	3	3	
65—271	m	174	25	170	n s
	n	4	3	3	
66—177	m	179	9	202	n s
	n	8	1	3	
All	m	171	21	191	n s

m* mean
n number of observati n
p** probability of differer en combined secretion rate and the sum of secretin and
rin secretion rates

TABLE V Secretion rate of protein (mg/min) in the period 0—10 min after stimulation with secretin (1 U/kg b w), gastrin (2 U/kg b w) or combined secretin gastrin

Dog no		Secretin	Gastrin	Combined	p**
64—208	m*	8 0	11 7	18 7	n s
	n	7	3	3	
65—231	m	9 5	18 3	21 5	<0 02
	n	6	2	3	
65—271	m	5 5	10 2	15 6	n s
	n	3	3	3	
66—177	m	8 2	4 7	10 3	n s
	n	7	3	3	
All	m	7 8	11 2	16 5	n s

m* mean
n number of observations
p** probability of difference between combined secretion rate and the sum of secretin and gastrin secretion rates

Discussion

In accordance with Gregory and Tracy (1964) the gastrin here applied exerted a stimulating effect on the pancreatic secretion of fluid, bicarbonate and protein Preshaw and Grossman (1965 c) failed to show this effect when using a crude gastrin preparation The divergency may be explained by the different gastrin preparations used

In the present investigation the combined effect of secretin and gastrin was demonstrated to be a summation, according to the definition given by Goodman and Gilman (1965) This finding is in agreement with the previous observation of Preshaw (1966)

The secretin preparation used contained some pancreozymin, and a potentiating effect on the secretin is to be expected (Brown *et al* 1967 Henriksen and Worning 1967) However, as the amount of pancreozymin injected was small (about 0 25 Crick Harper and Raper units/kg b w) this effect may only be slight Moreover, as the magnitude of the secretin-pancreozymin interaction was identical in the two groups of experimental data compared (Table III—IV) a potentiation between secretin and gastrin should appear if of any importance Similarly a hypothetical, mutual influence between gastrin and pancreozymin can not be expected to distort the results

Consequently it is concluded that the interaction of secretin and gastrin is not a potentiation but a simple summation

Brown, J C A A Harper and T Scratcherd Potentiation of secretin stimulation of the P Carl Petersen Foundation and the Danish Foundation for the Advancement of Medical Science

Grateful acknowledgement is made to *Leo* Pharmaceutical Products who supplied the eastrin preparation

References

Brown J C A A Harper and T Scratcherd Potentiation of secretin stimulation of the pancreas *J Physiol* (Lond) 1967 *190* 519—530

Fisher R A *Statistical methods for research workers* Thirteenth Ed Oliver and Boyd Edinburgh, London 1958 p 99—100

Goodman L S and A Gilman *The pharmacological basis of therapeutics* Macmillan New York, Third Ed. 1965 p 25—26

Gregory R A and H J Tracy, The constitution and properties of two gastrins extracted from hog antral mucosa *Gut* 1964 *5* 103—117

Henriksen F W Pancreatic response to repeated secretin stimulation in dogs *Acta physiol scand* 1966 *67* 214—218

Henriksen F W and H Worning The interaction of secretin and pancreozymin on the external pancreatic secretion in dogs *Acta physiol scand* 1967 *70* 241—249

Preshaw R M and M I Grossman Stimulation of pancreatic secretion by extracts of the pyloric gland area of the stomach. *Gastroenterology* 1965 a. *48* 36—44

Preshaw R M A R Cooke and M I Grossman Stimulation of pancreatic secretion by a humoral agent from the pyloric gland area of the stomach *Gastroenterology* 1965 b *49* 617—622

Preshaw R M and M I Grossman Comparison of subcutaneous and intravenous administration of pancreatic stimulants *Amer J Physiol* 1965 c *209* 803—810

Preshaw R M Stimulation of pancreatic secretion by gastrin extracts in Grossman M I *Gastrin* University of California Press Berkeley and Los Angeles 1966 p 309—323

Thomas J E An improved cannula for gastric and intestinal fistulas *Proc Soc exp Biol N Y* 1941 *46* 260—261

Van Slyke D D and J M Neill The determination of gases in blood and other solutions by vacuum extraction and manometric measurements *J biol Chem* 1924 *61* 523 573

Acta physiol. scand. 1969. 76. 73—81

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Control of Spontaneous Ventricular Fibrillation during Induced Hypothermia in Cats by Acute Cardiac Sympathectomy

By

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Received 2 August 1968

Abstract

NIELSEN, K C and CH OWMAN *Control of spontaneous ventricular fibrillation during induced hypothermia in cats by acute cardiac sympathectomy* Acta physiol scand 1969 *76* 73—81

17 adult cats were subjected to cardiac sympathectomy under nembutal anesthesia by bilateral excision of the cervical sympathetic chains between and including the superior cervical and stellate ganglia Immediately after operation, the anesthetized animals were cooled by submersion in an ice water bath Blood pressure, ECG, rectal temperature and respiration were continuously observed Angiotensin was administered intermittently to maintain the blood pressure at an adequate level at the lower body temperatures Artificial respiration was applied when indicated Under these conditions, only 2 out of 17 denervated animals died in ventricular fibrillation, at 23 and 18 4° C rectal temperature The remaining animals were cooled to 17 7—15° C and subsequently re warmed to normothermia without complications All of 17 unoperated control animals subjected to hypothermia in the same way developed ventricular fibrillation Cardiac noradrenaline determined fluorimetrically at the end of the experiments did not differ significantly between the two experimental groups The results of the present and earlier investigations indicate that hypothermia activates the sympathetic system through a central mechanism and that the cardiac sympathetic nerves represent an important factor for the spontaneous development of ventricular fibrillation a characteristic complication of induced deep hypothermia in homeothermic mammals

Ventricular fibrillation, usually the terminal event preceding death during induced deep hypothermia in homeothermic animals is the major complication limiting the clinical use of hypothermia in man

There is much evidence to indicate that the myocardium reaches a state of hyperexcitability when the body temperature is reduced and that this increased sensitivity is one important factor promoting the establishment of ventricular fibrillation (Brooks 1956 Hegnauer and Covino 1956 Hegnauer and Angelakos 1959, Hoffman 1959) However these changes are not in themselves sufficient to explain the characteristic onset of spontaneous ventricular fibrillation during hypothermia at about 21° C body temperature, there is reason to believe that some other factor is

involved as a 'trigger' mechanism for the hyperexcitable heart muscle at these body temperatures Results have indicated that both adrenaline and noradrenaline increase the susceptibility of the heart to ventricular fibrillation (Hoffman *et al* 1955), and adrenaline has been shown to rapidly induce fibrillation of the heart ventricle at temperatures around 20° C, as demonstrated both *in vivo* and *in vitro* (Berne 1954, Reissmann and Kapoor 1956) In view of these findings, it is notable that increased levels of circulating catecholamines have been measured during hypothermia (Brown and Cotten 1956, Holobut 1966)

In fluorescence microscopic investigations on the organization of the terminal sympathetic innervation in the heart, a characteristic difference in distribution of the cardiac adrenergic nerves has been demonstrated in hibernators compared with homeothermic mammals Thus few—if any—nerve terminals were found in relation to the myocardial muscle fibres in the ventricles of the hedgehog, thirteen lined ground squirrel and the bat, a varying amount of nerves innervated the cardiac blood vessels in these animals In contrast to this, a fairly rich adrenergic innervation *of both the blood vessels and the myocardium proper is found in homeothermic* animals such as cats guinea pigs, and rats (Nielsen and Owman 1965, 1968a) Moreover it has recently been shown that kittens having a not yet fully developed cardiac sympathetic innervation especially of the myocardium proper, are remarkably resistant to reduction in body temperature, and have been cooled down to about 17° C without developing ventricular fibrillation (Nielsen 1968)

On the basis of these findings a series of systematic investigations has been per- [illegible] on cats with the aim of finding ways of preventing the spontaneous ventricu- ar fibrillation occurring at reduced body temperature by interfering with various adrenergic mechanisms Furthermore the results have offered strong support for the hypothesis (see Nielsen and Owman 1965) that such mechanisms represent an important factor for the development of this serious cardiac complication of induced deep hypothermia. Thus after depleting the adrenergic nerves of their transmitter stores (Nielsen and Owman 1965, 1967b, 1968b), after blockade of the transmitter release from the adrenergic neurons (Nielsen and Owman 1968c), or after blocking the adrenergic β receptors (Nielsen and Owman 1965, 1966), cats have successfully been cooled to 18—15° C rectal temperature followed by re warming to normothermia without developing ventricular fibrillation

Control animals receiving no pretreatment constantly develop ventricular fibrillation at about 22—21 C during cooling under corresponding conditions

In the present study an attempt was made to delimit the hitherto obtained results to the level of the heart by interruption of the cardiac neuron link through acute sympathetic ganglionectomy

Material and Methods

The material includes 39 adult cats of either sex weighing between 2 5 and 5 kg The animals used for sympathectomy and those serving as unoperated controls were selected so that the means of the body weights in the two groups did not differ statistically from each other

Operation technique An operation procedure was chosen in which a maximum amount of

the sympathetic nerves to the heart could be severed bilaterally without necessitating thoracotomy, thus allowing the animals to breathe spontaneously after the operation Since in the cat about 25 % of the cardiac adrenergic nerves have been found to arise in the superior cervical ganglia (Nielsen *et al* 1968b), the cervical sympathetic chains were removed bilaterally between and including the superior cervical and stellate ganglia. There is reason to believe that this technique results in a pronounced degree of sympathetic denervation of the heart (Nielsen *et al* 1968a see also Discussion)

The animals were anesthetized with nembutal (30 mg/kg i p) and the vago-sympathetic trunk was identified at its position close to the common carotid artery at a level midway down the neck The sympathetic chain was carefully freed from the vagus nerve, and then followed distally to the stellate ganglion The sterno-cleidoid and major pectoral muscle were [illegible]

30 mg/kg of nembutal i p) were cooled by submersion in an ice water bath of 5—10° C temperature Arterial blood pressure ECG, respiration and rectal temperature were continuously observed and registered every 15 min (see Nielsen and Owman 1967) Artificial respiration was instituted by a pump respirator connected to an endotracheal T tube when spontaneous breathing became inadequate, usually at about 24° C rectal temperature Angiotensin (Hypertensin Ciba) was given to maintain a blood pressure above 60 mm Hg (see Haggendal 1965) This became necessary in most animals at body temperatures lower than about 23° C as soon as the systolic pressure fell below the 60 mm level 0 25—0 5 ml of angiotensin (dissolved in 0 9 % saline to a concentration of 1 μg ml) was slowly injected into the blood pressure catheter placed in the left common carotid artery The injection was repeated each time the blood pressure again fell below 60 mm Hg Angiotensin was chosen because it increases blood pressure through a direct effect on the vascular smooth muscle cells by a mechanism that does not involve the adrenergic receptors (Page and Bumpus 1961) Moreover, angiotensin increases sympathetic activity through a central mechanism (Bickerton and Buckley 1961), including an enhanced release of adreno-medullary catecholamines (Feldberg and Lewis 1961), it also has a ganglionic stimulatory effect (Lewis and Reit 1965) Administration of angiotensin could therefore be expected to offer further information concerning the importance of on one hand circulating catecholamines and on the other noradrenaline released from any intact cardiac sympathetic nerves on the development of ventricular fibrillation under the present experimental conditions

Animals that developed ventricular fibrillation were removed from the cooling bath and the experiment was thus discontinued Active cooling of the remaining animals was stopped at 18° C rectal temperature by replacing the ice water with warm water of 35—43° temperature The rectal temperature then continued to fall to 17 7—15° C before it reversed because of temperature gradients in the body The animals were subsequently re-warmed to normothermia.

Determination of noradrenaline After finishing the experiments the animals were bled and the heart was removed. The tissue was homogenized in perchloric acid and noradrenaline was determined fluorimetrically according to Bertler *et al* (1958) as modified by Haggendal (1963)

Student s t test was applied in all statistical operations

Results

The sympathectomized animals showed no or only slight, increase (up to 10 mm Hg) in blood pressure on submersion in the ice water The blood pressure then decreased progressively in the usual manner accompanying the reduction in body temperature In 9 of the 17 animals in this group the heart activity was perfectly rhythmic throughout the entire experiment (Table I) Four animals had short periods of cardiac arrhythmia during the cooling phase, in 2 animals, there were a few short intervals (lasting up to 15 sec) of ventricular tachycardia during which,

TABLE I Group of sympathectomized animals Body weight amount of angiotensin given during the experiment, lowest rectal temperature reached by the 15 surviving animals and the temperature at which 2 animals developed ventricular fibrillation and amount of cardiac noradrenaline measured at the end of each experiment (values not available for 2 of the animals) The comments on heart activity stand for both cooling and re warming

Body weight (kg)	Amount of angiotensin (μg)	Lowest temp (° C)	Cardiac noradrenaline (μg/g)	Cardiac activity
3 0	8 0	16 0	1 63	Rhythmic
3 0	7 5	17 4	1 37	Rhythmic
3 5	3 0	17 6	1 55	Rhythmic
5 0	0 5	17 4	—	Rhythmic
2 5	2 0	17 4	1 70	Rhythmic
4 2	2 5	17 2	1 37	Rhythmic
4 1	1 5	16 6	1 06	Rhythmic
3 9	1 5	16 5	—	Rhythmic
4 4	4 0	15 0	1 21	Rhythmic
3 5	1 0	17 7	1 33	Periods of arrhythmia
3 5	2 5	17 6	1 35	Periods of arrhythmia
3 4	2 5	17 0	2 10	Periods of arrhythmia
3 8	3 5	16 1	1 41	Periods of arrhythmia
4 5	4 5	17 2	1 50	Periods of tachycardia
[illegible]6	4 5	17 6	2 68	Periods of tachycardia
	4 0	18 4	1 20	*Ventricular fibrillation*
[illegible]	0	23 0	1 38	*Ventricular fibrillation*

however the blood pressure never fell below 30 mm Hg One of these 2 animals had an approximately 15 min period of cardiac standstill at the end of the cooling phase The heart activity and blood pressure picked up spontaneously a few minutes after re warming was started (Fig 1) Only 2 out of the 17 sympathectomized animals went into a continuous state of ventricular fibrillation (at 23 and 18 4° C rectal temperature respectively Table I and Fig 1) with no measurable blood pressure

The 15 animals that did not die in ventricular fibrillation reached 17 7—15° C rectal temperature before re warming towards normothermia began, this was continued without complications Spontaneous breathing started at about 25° C

All animals except one (fibrillating) had to receive angiotensin (0 5—8 μg per animal, Table I) to maintain the blood pressure above the 60 mm Hg level (see Material and Methods) at rectal temperatures below 25—21° C during cooling and at the beginning of the re warming period

The blood pressures rectal temperatures and cooling times for the group of sympathectomized animals are summarized in Fig 1

When cooling of the unoperated control animals was started, the blood pressure

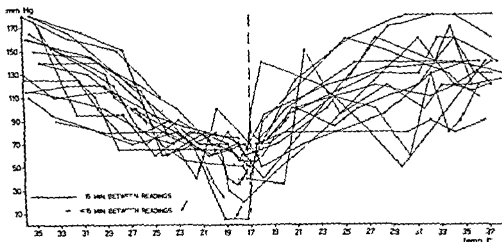


Fig 1 Arterial blood pressure (ordinate) rectal temperature (abscissa) and cooling time of the 17 sympathectomized animals Two animals died in ventricular fibrillation at 23 and 18 4 C (arrows) One animal had a 15-min period of cardiac standstill at the end of cooling between about 19 and 17° C rectal temperature Fifteen of the animals were re-warmed to normothermia Lowest rectal temperatures of these 15 surviving animals are given in Table I

showed a short lasting increase by more than 45 mm Hg All animals in the control group developed ventricular fibrillation at a mean rectal temperature of 21 3 ± 0 4° C This was significantly higher (P<0 001) that the lowest rectal temperature (17 0 ± 0 03 C) of the surviving animals in the operated group 8 of the animals were given intermittent injections of angiotensin (2—6 μg per animal) starting at approximately the same body temperature as in the operated animals Artificial respiration was applied at 24 5—22° C rectal temperature The cooling curves had the same principal appearance as in a previous identically treated group of control animals (see Nielsen and Owman 1968c)

Cardiac noradrenaline was *determined fluorimetrically in 5 non-cooled animals* The mean value of the amine concentration (1 60 ± 0 15 standard error of the mean) in these animals did not differ statistically (P>0 05 from the noradrenaline *concentration in the hearts of the operated group* (1 52 ± 0 10)

Discussion

A recent series of investigations on hibernators and homeothermic animals have of fered strong support for the hypothesis that adrenergic mechanisms play an im *portant role in the development of ventricular fibrillation* at reduced body tem perature Histochemical analysis of the organization of the adrenergic innervation to the heart has revealed that remarkably few—if any— sympathetic ne *occur in relation to the muscle cells of the ventricles in hibernat* myocardium proper (as well as the cardiac circulation) is supp

well-developed sympathetic innervation in homeothermic animals (Nielsen and Owman 1965, 1968a) Differential depletion of the myocardial catecholamine stores with prenylamine (Nielsen and Owman 1965, 1967a and b) efficiently prevents the development of ventricular fibrillation in cats cooled to 17 8—17° C rectal temperature This protective effect of prenylamine is reversed during infusion of noradrenaline which is taken up into, and then probably released from, the cardiac sympathetic nerve terminals (Nielsen and Owman 1967b) Hypothermic ventricular fibrillation is also controlled after more extensive depletion of the catecholamine stores with reserpine (Nielsen and Owman 1968b) In accordance with these findings, it is notable that kittens, having a not yet fully developed sympathetic innervation to the heart, are conspicuously resistant to reduction in body temperature (Nielsen 1968) Moreover, blockade of the transmitter release from the adrenergic nerve terminals (Nielsen and Owman 1968c) as well as blockade of the adrenergic β receptors (Nielsen and Owman 1965, 1966) were found to offer an effective protection to adult cats against the development of hypothermic ventricular fibrillation

The very low incidence of ventricular fibrillation after sympathectomy in the present experiments supports the assumption that the adrenergic innervation in the heart, rather than other parts of the sympatho-adrenal system is the major factor initiating fibrillation in the hyperexcitable ventricular myocardium This agrees with recent findings on isolated heart preparations in which ventricular fibrillation could induced by tyramine at reduced but not at normal, temperatures and that the rillation was reversed into rhythmic cardiac activity by the addition of a specific β-adrenergic blocking agent without quinidine like side effects (Falck *et al* 1968b) Therefore it seems of little significance for the outcome of the present experiments that hypothermia in itself increases the level of circulating catecholamines (Brown and Cotten 1956 Holobut 1966) and that administration of angiotensin activates the sympathetic system (Bickerton and Buckley 1961) and releases catecholamines from the adrenal medulla (Feldberg and Lewis 1961) However it should be remembered that the operation procedure used including excision of the superior cervical ganglia also denervates the pial circulation of the brain (Nielsen and Owman 1967c) This has turned out to result in a marked dilation of the pial vessels (Falck *et al* 1968a) which may be one reason for the surprisingly good general condition of the sympathectomized animals during the hypothermia (cf Nielsen 1961)

The present findings are well comparable to the results obtained by Shumacker *et al* (1956) who induced ventricular fibrillation in dogs cooled to about 27° C rectal temperature by occlusion of the caval veins together with right ventriculotomy Ventricular fibrillation could under these circumstances be prevented by bilateral sympathetic denervation of the heart The reason for their lower incidence of ventricular fibrillation with animals in which the vagi were divided below the nodose ganglion might be explained by the presence of adrenergic fibres from the superior cervical ganglion running in the vagus nerve to the heart (Nielsen *et al* 1968b)

The only reliable technique producing a total cardiac sympathectomy seems to be that of cardiac autotransplantation (see Cooper 1966) Since no intra-cardiac adrenergic ganglion formations have been found in the heart (Ehinger *et al* 1967) it can be assumed that any residual catecholamines present in the heart after autotransplantation are stored not in nerves but in "chromaffin" cell formations (Jacobowitz 1967, Nielsen and Owman 1967b) The technique used in this study is more simple, it leaves the animal in good condition for the immediately following hypothermia experiment, and a very pronounced degree of denervation is achieved in many of the animals as judged from the level of residual noradrenaline 3 days after operation (Nielsen *et al* 1968a) Cervical sympathectomy in the dog reduces cardiac noradrenaline to one third of the normal level, and there is reason to believe that also the stellate ganglia substantially contribute to the adrenergic innervation of the heart (Goodall and Kirshner 1956) The two animals dying in ventricular fibrillation and the two animals having short periods of ventricular tachycardia would well correspond in number with the rate of incomplete denervation seen in the previously mentioned study (Nielsen *et al* 1968a) In the remaining animals, the denervation has obviously been sufficiently complete to prevent the development of ventricular fibrillation during hypothermia to rectal temperatures between 17 7 and 15° C

It was found in earlier experiments that hypothermia *per se* reduces the noradrenaline content in the sympathetic nerve terminals of the heart (Nielsen and Owman 1967b, Nielsen *et al* 1968a), probably as a consequence of an enhanced transmitter release (Goldstein and Nakajima 1966) It is therefore of interest that in the sympathectomized animals there was only a slight increase in blood pressure upon submersion in the cooling bath as compared with the intact animals Further, the cardiac noradrenaline concentration at the end of the experiments did not differ from the normotherms whereas cooling of intact animals has been shown to result in a significant reduction in cardiac noradrenaline compared with corresponding normothermic control animals (Nielsen and Owman 1967b Nielsen *et al* 1968a) This offers further evidence in support of the view that a substantial degree of cardiac denervation has been achieved in the operated animals It also indicates that an intact sympathetic innervation to the heart is indispensable for an increased output of noradrenaline from the sympathetic nerve terminals during hypothermia In view of this it is understandable that the incidence of ventricular fibrillation was very low in the present experiments despite the fact that angiotensin, given to maintain the blood pressure at an adequate level during reduction in body temperature markedly activates the sympathetic system (Bickerton and Buckley 1961) and has a prominent ganglionic stimulatory action (Lewis and Reit 1965) In a previous investigation (Nielsen *et al* 1968a) animals were sympathectomized according to the procedure used in the present study but cooling was not performed until 2—3 days later in order to allow the transmitter to disappear from the nerve terminals Under these conditions however the incidence of ventricular fibrillation was not significantly reduced The discrepancy between the present and the previous results seems

to be well explained by the rapid development of supersensitivity (Langer *et al* 1967) of the myocardial receptors, particularly towards the noradrenaline released from those adrenergic nerves escaping denervation in the heart (see Nielsen *et al* 1968a)

Supported by grants from the Association for the Aid of Crippled Children, New York and from Sven Eric and Anna Lisa Lundgren s Foundation

References

BERNE R M The effect of immersion hypothermia on coronary blood flow *Circulat Res* 1954 *2* 236—242

BERTLER A, A CARLSSON E ROSENGREN and B WALDECK A method for the fluorimetric determination of adrenaline noradrenaline and dopamine in tissues *Kungl Fysiogr Sallsk Lund Forh* 1958 *28* 121—123

BICKERTON R K and J P BUCKLEY, Evidence for a central mechanism in angiotensin induced hypertension *Proc Soc exp Biol* (N Y) 1961 *106* 834—836

BROOKS C M Hypothermia and the physiology of cardiac excitability *In The Physiology of Induced Hypothermia* Ed by Dripps R D, Nat Acad Sci Nat Res Counc, Washington 1956 287—301

BROWN T G and M V COTTEN Evaluation of factors enhancing cardiac force during hypothermia *Fed Proc* 1956 *15* 405

COOPER TH Surgical sympathectomy and adrenergic function *Pharmacol Rev* 1966 *18* 611—618

EHINGER B B FALCK and B SPORRONG Adrenergic fibres to the heart and to peripheral vessels *Bibl anat* (Basel) 1967 *8* 35—45

FALCK B K C NIELSEN and CH OWMAN, Adrenergic innervation of the pial circulation *Scand J clin Lab Invest* 1968a Suppl 102

FALCK B K C NIELSEN CH OWMAN H PERSSON and B SPORRONG 1968b In preparation

FELDBERG W and G P LEWIS The action of peptides on the adrenal medulla Release of adrenaline by bradykinin and angiotensin *J Physiol* (Lond) 1964 *171* 98—108

GOLDSTEIN M and K NAKAJIMA The effect of disulfiram on the biosynthesis of catechol amines during exposure of rats to cold *Life Sci* 1966 *5* 175—179

GOODALL M and N KIRSHNER Effect of cervico-thoracic ganglionectomy on the adrenaline and noradrenaline content in the mammalian heart *J clin Invest* 1956 *35* 649—656

HAGGENDAL E Blood flow autoregulation of the cerebral grey matter with comments on its mechanism *Acta neurol scand* 1965 Suppl *14* 104—116

HAGGENDAL J An improved method for fluorimetric determination of small amounts of adrenaline and noradrenaline in plasma and tissues *Acta physiol scand* 1963 *59* 242 254

HEGNAUER A H and E T ANGELAKOS Excitability properties of the hypothermic heart *Ann N Y Acad Sci* 1959 *80* 336—347

HEGNAUER A H and B G COVINO Myocardial irritability in experimental immersion hypothermia *In The Physiology of Induced Hypothermia* Ed by Dripps R D Nat Acad Sci Nat Res Counc Washington 1956 327—340

HOFFMAN B F Hypothermia and vulnerability *Ann N Y Acad Sci* 1959 *80* 346—350

HOFFMAN B F A A SIEBENS P F CRANEFIELD and C M BROOKS The effect of epinephrine and norepinephrine on ventricular vulnerability *Circulat Res* 1955 *3* 140—146

HOLOBUT W Catecholamine and histamine content of peripheral blood during hypothermia *Acta Physiol Hung* 1966 *29* 383

JACOBOWITZ D Histochemical studies of the relationship of chromaffin cells and adrenergic nerve fibers to the cardiac ganglia of several species *J Pharmacol exp Ther* 1967 *158* 227—240

LANGER S Z P R DRASKOCZY and U TRENDELENBURG Time course of the development of supersensitivity to various amines in the nictitating membrane of the pithed cat after denervation or decentralization *J Pharmacol exp Ther* 1967 *157* 255—273

LEWIS G P and F REIT Stimulation of the superior cervical ganglion of the cat by angiotensin and bradykinin *J Physiol* (Lond) 1965 *176* 28P

NIELSEN, K. C. Signs of hypoxia caused by hyperventilation during hypothermia. *Acta med scand* 1961 *170* 765—776
NIELSEN K. C. Possible relation between the degree of cardiac adrenergic innervation and the resistance to hypothermic ventricular fibrillation in young cats *Acta physiol scand* 1969 *76* 1—9
NIELSEN K. C. and CH OWMAN On the control of ventricular fibrillation during hypothermia. *Communic Dept Anat Univ Lund* 1965 6 1—13
NIELSEN K. C. and CH OWMAN Control of ventricular fibrillation by a *beta* adrenergic blocking agent (INPEA) during induced hypothermia in cats *Life Sci* 1966 *5* 1611—1623
NIELSEN K. C. and CH OWMAN Differential amine depletion from cardiac adrenergic nerves by Segontin *Experientia* (Basel) 1967a. *23* 203—204
NIELSEN K. C. and CH OWMAN Control of ventricular fibrillation during induced hypothermia in cats after differential depletion of cardiac catecholamines stores with prenyl amine (Segontin) *Circulat Res* 1967b *21* 45—58
NIELSEN K. C. and CH OWMAN Adrenergic innervation of pial arteries related to the circle of Willis in the cat. *Brain Res* 1967c *6* 773—776
NIELSEN K. C. and CH OWMAN Difference in cardiac adrenergic innervation between hibernators and non hibernating mammals *Acta physiol scand* 1968a Suppl 316
NIELSEN K. C. and CH OWMAN Effect of reserpine on the spontaneous ventricular fibrillation developing during induced deep hypothermia in cats. *Arch int Pharmacodyn* 1968b In press
NIELSEN K. C. and CH OWMAN Control of ventricular fibrillation during induced hypothermia in cats after blocking the adrenergic neurons with bretylium *Life Sci* 1968c. *7* 159—168
NIELSEN K. C. CH OWMAN and E ROSENGREN Effect of cardiac catecholamine depletion through sympathectomy on spontaneous ventricular fibrillation during induced hypothermia in cats. *Experientia* (Basel) 1968a In press
NIELSEN K. C. CH. OWMAN and M SANTINI Anastomosing adrenergic nerves from the sympathetic trunk to the vagus at the cervical level in the cat. *Brain Res* 1968b In press
REISSMANN K. R and S KAPOOR Dynamics of hypothermic heart muscle (heart lung preparation) *Amer J Physiol* 1956 *184* 167—170
SHUMACKER H B A RIBERI R. D BOONE and H. KAJIKURI Ventricular fibrillation in the hypothermic state IV The role of extrinsic cardiac innervation *Ann Surg* 1956 *143* 223—229

Acta physiol. scand. 1969. 76. 82—92

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Colour Specific Features of Visual Cortical Responses in Man Evoked by Monochromatic Flashes

By

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Received 5 August 1968

Abstract

CIGÁNEK, L. and D. H. INGVAR. *Colour specific features of visual cortical responses in man evoked by monochromatic flashes.* Acta physiol. scand. 1969. 76. 82—92.

In 16 normal human subjects a study was made of averaged responses in the occipital regions to monochromatic flashes of 15 different wavelengths with equal energy. The responses to monochromatic flashes were found to have the same principal components as those previously hown in responses to white light. With low intensity flashes of equal energy, the latencies of ll components were shortest in the middle range (514—576 mμ), longer in the blue and longest in the red end of the spectrum. With high intensity stimuli the latencies were in general shorter and did not vary with wavelength. The high variability of the amplitudes of the evoked responses made it difficult to correlate amplitude with wavelength. However some individual curves as well as the average amplitude/wavelength curve for the whole group of 16 subjects showed partly significant peaks at 453—475, 551 and at 615 mμ, a finding which appears to support the existence of a trichromatic colour receptor mechanism in man. One group of the subjects showed a strikingly stable form of the evoked responses but varying amplitudes of separate components at different wavelengths. Another group showed very stable forms of the responses which appeared specific to wavelength.

There is still a remarkable disproportion between the abundant knowledge of the colour reception mechanism of the retina and the lack of information on the transmission of colour messages to the visual centres of the brain. A number of recent studies suggest that there are probably three types of retinal colour receptors in primates with sensitivity maxima in the blue, the green and the red (Stiles 1959, Brindley 1960, Rushton 1961) as well as in lower species (Granit 1945, MacNichol *et al.* 1961). These findings have in general been taken to show that retinal colour reception may operate in accordance with the trichromatic theory of Young (1801).

Several studies have indicated that responses to monochromatic light in the visual centres may show certain colour specific features (Adrian 1941, Motokawa *et al.* 1952, Lennox and Madsen 1955, Madsen and Lennox 1955, Lennox 1956, 1958a,

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b, 1962, De Valois *et al* 1958, 1962, Mac Nichol and Svaetichin 1958, Ingvar 1959, Hubel and Wiesel 1960, Monnier 1963, Monnier *et al* 1964, Alpern 1965, Shipley *et al* 1965, Siegfried *et al* 1965, Naka 1966, Wiesel and Hubel 1966, Herberg *et al* 1967, Jung 1964, Monnier and Rozier 1967, Regan 1967, Sanders and Liske 1967) Some of these studies suggest that the visual messages to the brain are processed into three "contrast" systems for red green, blue yellow, and for black-white, according to the classical Hering (1895) opponent-colour theory (Mac Nichol and Svaetichin 1958, De Valois *et al* 1958, 1962, Hubel and Wiesel 1960, Wiesel and Hubel 1966) Regarding the arrival of colour messages to the visual cortex, Ingvar (1959), Lennox (1962), and Wiesel and Hubel (1966), amongst others, have shown that cortical neurons or groups of neurons may exhibit both narrow spectral sensitivity peaks and opponent colour characteristics Many of the studies mentioned especially those undertaken in man, are, however, rather incomplete and do not give a systematic information about the spectral characteristics of colour messages to the visual centres of the brain

As pointed out by one of us (Ingvar 1959), the study of evoked potentials in the visual cortex appears to offer specific advantages for a 'remote' investigation of retinal messages on the wide visual fields Thus, on diffuse illumination, a small electrode recording from the visual cortex may pick up a differentiated message from only a small group of retinal receptors Thus, details may be revealed which are not seen in the mass responses (electroretinogram etc) from primary visual pathways

In the present study in normal human subjects, we have used high intensity monochromatic flashes, calibrated to have an equal energy Visual evoked responses to the flashes were recorded from the occipital region and their latencies amplitudes and form were correlated with the wavelengths used It will be shown that distinct differences in the parameters studied were found when different colours were used Furthermore, these differences showed certain systematic changes which indicated the presence of sensitivity peaks within the spectrum

Materials and methods

[illegible]

During the colour stimulation the subject was seated on a straight chair with the chin and forehead resting on a conventional ophtalmological support The right pupil was maximally dilated by Cyclogyl® and in front of it at a distance of 3—4 cm, a thin milk glass was placed covering a circular window in which the monochromatic flashes appeared The diameter of the window was 4 cm The subjects were asked to look straight ahead into the window and before each series of flashes a reminder to keep the gaze in the desired direction was given

The influence of retinal adaptation was not investigated systematically Five subjects were however studied in darkness following a period of dark adaptation for 20 min The remainder were studied at two different levels of background illumination a higher and a lower one The cortical responses were not appreciably influenced by such variations in the background illumination They were found to be much more dependent upon the intensity of the flashes

The stimulus In Fig 1 a schematic drawing is seen of the optical equipment A high intensity Xenon lamp (Osram XBO 162) was employed It has an approximately linear spectral emission within the range 400—700 mμ It was housed in a ventilated solid metal box The

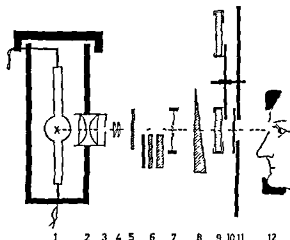


Fig 1 *Diagram of the optical system* 1/ High intensity Xenon lamp 2/ Condensor with lenses 3/ Small hole with a diameter of 2 mm 4/ Condensor lenses 5/ Heat filter 6/ Neutral filters 7/ Shutter 8/ Neutral wedge filter 9/ Set of 15 interference filters mounted on a circular plate 10/ Milk glass 11/ Metal shield with window 12/ Head support for the subject

light from the lamp was condensed by quarz lenses which had an equal spectral absorption within the part of the spectrum used In the optical axis there followed a heat filter (Balzers Lichtenstein(with a very sharp cut off at wavelengths above 700 mμ a holder with a set of neutral filters (American Optical Co) with densities of 0 3 0 6 0 9 and 1 2 *log* units a conventional camera shutter (Leitz Germany) for 500 ms flashes of the light beam a wedge of neutral glass covering 2 log units for adaptation of the coloured flashes to an equal energy In some studies an extra neutral filter was used which only let through 27 per cent of the light beam (expressed in thermoelement units as explained below)

The interference filters These filters (Balzers Lichtenstein) had their narrow transmission peaks at the following wavelengths 414 433 453 475, 491, 514 533 551 576 595 615 631 658 675 and 691 mμ The peaks as well as the spectral transmission curves for all filters were measured in a spectrophotometer These curves showed for all filters that the transmission peaks had a width of only 15—19 mμ at a transmission half of that at the peaks given above

Finally there was a 2 mm thick milk glass in front of the opening facing the right eye of the subject Its spectral absorption was approximately linear over the range of wavelengths used The flashes were presented randomly with average intervals of about 1 sec

Calibration The monochromatic light flashes were calibrated to equal energy by placing a vacuum thermoelement (System Haase) at the end of the optic system in the position of the subject s eye During the calibration the shutter was opened and the milk glass was taken away The monochromatic light beam was then focused onto the small sensitive surface of the thermoelement by means of a positive lens The potential generated by the thermoelement was recorded by means of a sensitive voltmeter (Hewlett Packard microvoltammeter type 425 A) Three energy calibrations were made before the beginning of the study and one after In the last one it was found that the use of the lamp (during 73 hrs) had diminished the intensity by 26 per cent at 414 by 20 per cent at 433 by 16 5 per cent at 453 by 9 per cent at 475 and by 7 5 per cent at 491mμ The other wavelengths showed intensities identical with those at the beginning of the study which altogether lasted about two months

In all the subjects the separate wavelengths were used in random order avoiding neighbouring colours in the spectral range In two subjects however the wavelengths were used in a sequence from the blue to the red end of the spectrum

Recording technique Routine metal EEG electrodes mounted with bentonite paste and adhesive tape were used in the placements O_2 and P_2 according to the 10—20 system (Jasper 1958) They were connected to the input of a preamplifier (Tektronix 122) in such a way that the relative negativity of the occipital electrode resulted in an upward deflection on the record For further amplification the amplifiers of a dual beam oscilloscope (Tektronix 502) were used The sweep was triggered by a small contact connected to the shutter The preamplifiers were used with a setting of the low and high frequency cut-off at 8 and 50 cps respectively

The signals from the vertical plates of the oscilloscope were fed into the input of a Mnemotron CAT 1000 The signals during the first 500 ms following the onset of the light stimulus were averaged 50 or 100 responses were usually recorded and averaged in each

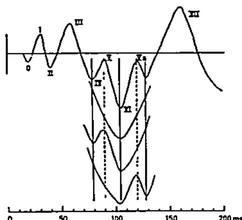


Fig 2 *Diagram of the averaged normal human response in the occipital region evoked by monochromatic stimulation with a coloured flash* Electrodes in placements O_2 and P_2 according to the 10—20 system The negativity of the electrode O_2 resulted in an upward deflection of the record The middle positive part of the response often showed varying smaller deflections superimposed on the larger positive wave.

series and for each wavelength The averaged responses were written on a X-Y plotter (Mosley X Y recorder, Model 2D2) with a time scale of 1 ms/cm and an amplitude scale of 1—2 µV/cm

Since the amplitude and other characteristics of evoked responses are known to vary with the level of wakefulness of the subject a control EEG was recorded (Grass EEG machine IV B) in all subjects from the same occipital electrodes (O_2—P_2) The EEG was monitored during the whole stimulation period and, in a few instances, when signs of drowsiness were seen, the subjects were aroused verbally

Results

1 *General characteristics of the responses*

In all but a negligible number of instances, all the series of monochromatic flashes in all the subjects, gave rise to clear-cut average evoked potentials In spite of the gross stimulation technique and the varying light adaption conditions very distinct general similarities between the responses recorded and the "classical" visual evoked response to white light (Ciganek 1961 a, b) could be recognized Thus as seen in Fig 2, all of the components III to VII (Ciganek 1961 a, b Gastaut *et al* 1967) could be identified in the average curves The negative peak III was always followed by a pronounced positive deflection (IV—VI) which consisted either of a smooth single positive wave, or a positive wave with various superimposed smaller waves (V and V a) This positive complex was always followed by a negative wave VII Waves 0 I, and II appeared rather exceptionally in a clear-cut form

In the following analysis peaks III IV V V a, VI, and VII were especially taken into consideration

2 *Latency of the responses related to wavelength*

Two distinct latency patterns were observed of the evoked responses as a function of wavelength one pertaining to low and the other to higher intensities of stimulation

The low intensity latency pattern had the following characteristics At equal energy of the stimuli the latency was found to be shortest in the middle range of the spec-

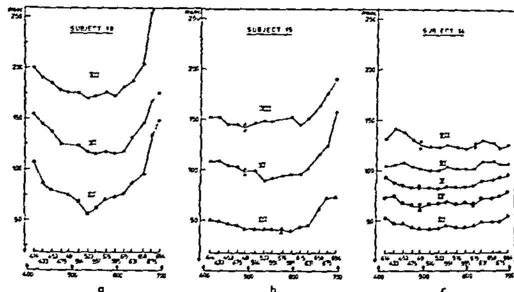


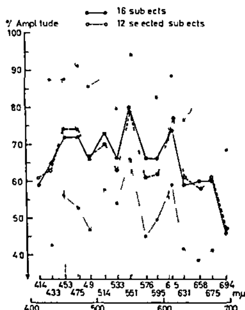
Fig 3 Latency of the averaged visual cortical response to monochromatic flashes related to wavelength of the stimulus Three different subjects three different stimulus intensity levels. Roman numerals indicate response components measured (Fig 2) In some cases the latency was measured from two different averaged responses and their mean was used in the curve. Dashed lines indicate lack of clear-cut response component Dashed lines with arrows at the right end of some curves denote probable trend of the curve in cases where the response at 694 mμ was small and difficult to measure Note that the longest latencies were found at the lowest intensity level Subject 10 a the shortest—and colour insensitive—latencies were found at the highest intensity level Subject 14 c Subject 15 (b represents an intermediate at an intermediate intensity level

trum in the 514—576 mμ range whereas the latency was only about half of that in the blue and perhaps only one quarter in the red end (Fig 3 a) This latency variation was found in all components of the cortical response

The high intensity latency pattern on the contrary was in its most pronounced form characterized by a *lack* of variation with wavelength It was seen when the flashes were given without the use of neutral filters (Fig 3 c) In many cases with moderate light stimulus intensities intermediate patterns were seen in which there was usually longer latencies in the red end of the spectrum and about equal latencies in the blue end Fig 3 b In general the two main latency patterns *could* always be identified but occasionally other superimposed patterns within parts of the spectrum were seen. These were not numerous and they were not investigated systematically

3 *Amplitude of the responses related to wavelength*

It is well known that the amplitude of cortical evoked potentials varies considerably (*cf* Jonkman 1967) This variation is for unknown reasons more pronounced in some subjects than in others For the present peak to peak amplitude measurements it was often necessary to use different components of the response even in the same



vertical lines see text)
(Statistical significances of some positive negative peak differences in the curve of all the 16 subjects (t test)

414—453 mμ p<0 02
475—491 mμ p<0 25 475—533 mμ p<0 05
491 514 mμ p<0 25
514 533 mμ p<0 02
533—551 mμ p<0 01
551 576 mμ p<0 05
595—615 mμ p<0 25 for 12 subjects p<0 10
615—631 mμ p<0 002
615—694 mμ p<0 001)

subject since not all components could be identified for all colours in all subjects The amplitude most often measured was the IV—VII peak to peak distance (10 cases) The others were VI—VII and III—IV each in two cases and V—VI and III—IV each in one case

In Fig 4 an average curve is seen for all subjects showing the spectral amplitude variations Individual curves were scaled t gether with the 100 per cent value placed at the wavelength at which maximal amplitude was recorded The variations at each wavelength have been indicated as the S D of the respective percentual value (dotted lines) It is obvious that there were as expected very large variations and that a distinct pattern can only vaguely be recognized in the diagram However there were

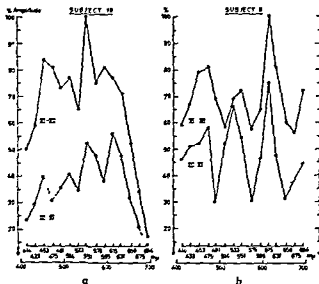


Fig 5 Two types of amplitude/wavelength curves plotted from occipital response to equal energy monochromatic flashes Amplitude scale as in Fig 4 In each subject two different peak to peak amplitudes were measured. Roman numerals indicate components measured The dashed lines connect values obtained from responses which were difficult to measure In subject 10 (a) a lower intensity level was used than in subject 8 (*b*)

ther suggestive peaks at 453—475 at 551, and at 615 mμ There was also a smaller ak at 514 and a questionable one at 675 mμ

An inter wavelength significance analysis of the curve of Fig 4 gave the following result The peak at 551 mμ was statistically significant at the 1 per cent and the 5 per cent level when compared with the adjoining wavelengths The rising slope of the peak at 453 475 mμ was clearly significant at the 2 per cent level Concerning its descending slope the difference was significant at the 5 per cent level In this connection and taking the significances of differences 491—514—533 mμ see text of Fig 4 into consideration the significance of the peak at 514 mμ appears to be highly dubious Finally the peak at 615 mμ was highly significant at the 0.2 per cent level when compared with the 631 mμ values The other rising slope was not significant for the average of all 16 subjects but the same difference (595 615 mμ) for 12 selected subjects showed a slight significance at the 10 per cent level The small peak at 675 mμ was not significant

It was attempted to identify a low and a high intensity amplitude pattern as for latency see above However such patterns were never encountered as distinctly as for the latencies Only a few of the subjects showed patterns which suggested two main types Fig 5 The first, possibly a *low intensity amplitude pattern* (Fig 5 a was characterized by higher amplitudes in the middle part of the spectrum and lower in the blue and especially in the red end Superimposed upon this curve various smaller peaks were sometimes recorded which however often coincided with the peaks of the average amplitude diagram in Fig 4 In the *high intensity amplitude pattern* usually a very complex amplitude/wavelength relationship was observed yielding curves with variable peaks which however often showed preferential spectral placements of the same type as seen in the average curve (Fig 4) Such an example is given in Fig 5 b

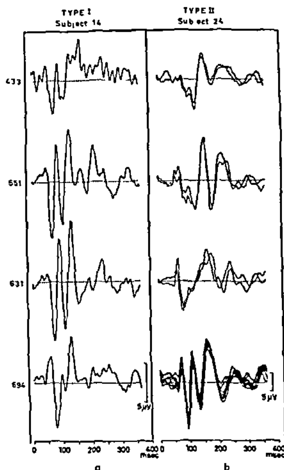


Fig 6 *Form of cortical response to monochromatic flashes related to wavelength* In type I the form of the response was uniform throughout the spectrum but the amplitude varied In type II specific forms for different wavelengths were recorded The stability of the specific forms is shown in the superposition of several averaged responses obtained at irregular intervals during the study in subject 24 The whole recording period in this case lasted 145 minutes At 694 mμ 7 averaged responses were recorded which all showed a strikingly identical form.

4 *Form of the responses related to wavelength*

It is not possible to give a full account of all the different forms of responses encountered since the separate components varied so much In general however there were two extreme cases Group I (Fig 6 a) showed a strikingly similar and stable form but varying amplitudes of the separate components at the different wavelengths In this group the most definite three peaked amplitude curves (according to Fig 4 and 5 b) were seen Group II (Fig 6 b) showed distinctly different forms of the responses which appeared specific to wavelength In this group the form changed so much that a uniform amplitude measurement based upon the identical components in all colours could not be carried out Fig 6 shows typical responses pertaining to Group I and II from two subjects at wavelengths 433 551 631 and 694 mμ

Discussion

The main objective of this study was to establish whether evoked potentials from the visual cortex in man show any characteristics at all which can be related to the wavelength of a flash stimulus Previous studies of this problem have yielded contradictory or even negative results (Monnier 1963, Monnier *et al* 1964 Alpern 1965 Shipley *et al* 1965 Siegfried *et al* 1965, Monnier and Rozier 1967, Regan 1967 Sanders and Liske 1967) Any study of visual evoked responses in man even when using an averaging technique is hampered by the fact that such responses vary extensively due to factors which cannot at all or only to a limited extent, be controlled Among these factors are the pre retinal absorption of the eye media the direction of the gaze variations in light adaptation anatomical variations of the visual cortex in relation to the recording points extracranially Furthermore the responses vary with the background EEG in general and interfere with the alpha activity in particular This interference may also change in the same subject during a longlasting study due to habituation to the experimental situation slight variations in awareness attention emotional state etc In view of this impressive list it is surprising that any *systematic changes of the responses with wavelength were obtained at all However* the findings were limited and only a few comments seem warranted on how the results relate to present knowledge on colour vision

The first conclusion is that the cortical response to monochromatic light did not show any essential new components in addition to those known since long pertaining to the response to white light (Ciganek 1961 a b) The changes with wavelength appeared as quantitative variations—especially as to amplitude—of the same components which in their turn under certain not fully understood conditions may create responses with a specific form for different colours (Fig 6 b) The possibility that such responses represent messages from cone rich foveal parts of the retina which use the same pathways and therefore express specific coding for separate colours would merit further study

The next result which appears clear cut was the finding that there were two latency wavelength patterns The low intensity pattern with a rounded peak in the middle range of the spectrum suggests its dependence on a scotopic rhodopsin related mechanism At high intensity levels another possibly photopic mechanism *was present which operated in a fundamentally different way the high intensity* monochromatic messages arriving with practically identical latency for all wavelengths At this intensity level other response characteristics such as amplitude and/or form appeared to carry the information about the colour Concerning amplitude it has been emphasized above that the variability of the responses sets definite limits for conclusions to be drawn Thus no definite low and high intensity patterns i e a scotopic and photopic pattern were identified

The mean spectral sensitivity curve (Fig 4) should be accepted with reservation due to the numerous factors which might have influenced it However there were general similarities between the sensitivity peaks found in it and similar peaks found in other studies MacNichol (1964 identified photopigments in the goldfish retina

with a maximal absorption at 455, 530, and at 625 mμ Furthermore, Granit (1945, 1948) showed the presence of 'modulators" with maximal sensitivity at 440—460 mμ, at 520—540 mμ and at 580—600 mμ Finally, Ingvar (1959) demonstrated spectral sensitivity peaks in the cat by means of a technique similar to that used here, also at 450—470 550 and 610 mμ for high intensity monochromatic flashes It therefore does not seem unlikely that the spectral sensitivity peaks observed in the present study in the blue green and red, may be related to a trichromatic colour discriminating mechanism in normal man

The present study has clearly shown that it is not yet possible to define the ideal experimental conditions for measurement of responses to monochromatic light in the visual cortex in man Apparently, the criteria studied here, latency, amplitude, and form of averaged evoked response, do not suffice for a sensitive analysis It is mainly the form sometimes highly correlated to wavelength (Shipley *et al* 1965, Sanders and Liske 1967) which escapes an exact and objective description and measurement Other methods including computer techniques will probably have to be used to increase the resolution of the evoked potential analysis in the study of colour vision

Swedish Medical Research Council (contracts ... indation Stockholm and from AB Akerlund ... alyser was kindly placed at our disposal by ... gave valuable assistance with the electronic work

In a recent study of the visually evoked occipitogram in man Shipley *et al* (Vision Res 1968 *8* 409—431) have shown that the waveform of occipital responses to 16 different steps within the visible spectrum varied w... technique instead of energy ... that Shipley *et al* were abl... absence of this coding in oth... and group 2 of the present study

References

ADRIAN E D Visual responses in the cat and monkey *J Physiol* (Lond) 1941 *100* 9 P

ALPIERI R The human electroretinogram the e-waves and the vision of colours *C R Soc Biol* (Paris) 1965 *159* 2362—2366

BRINDLEY G S Physiology of the retina and the visual pathway Arnold London and Williams and Wilkins Baltimore (1960)

CIGANEK L ... treizantwort der menschlichen Hirnrinde ... lava 1961a 151 p

CIG... to light stimulus in man. *Electroenceph...*

DE VALOIS R L C J SMITH S T KITAI and A J KAROLY Response of single cells in monkey lateral geniculate nucleus to monochromatic light *Science* 1958 *127* 238—239

DE VALOIS R L G H JACOBS and A E JONES Responses of single cells in primate red green color vision system. *Optik* (Stuttgart) 1962 *20* 87—98

DE VALOIS R L and A E JONES Single cell analysis of the organization of the primate color vision system In The Visual System *Neurophysiology and Psychophysics* 178—191 Ed Jung R. and Kornhuber H Springer Berlin 1961

GASTAUT H F BOSTEM A WALTREGNY R POIRE and H REGIS *Les activites electriques cerebrales spontanées et evoquees chez l'homme* Gauthier Villars (Paris) 1967 236 p

GRANIT R The colour receptors of the mammalian retina *J Neurophysiol* 1945 *8* 195—210

HERBERG K P F H DUFFY and C T LOMBROSO Visual evoked potentials of monochromatic stimulation in controls and photo-sensitive epileptic subjects In *American electroencephalographic society* Twenty ...

Hubel D H and T N Wiesel, Receptive fields of optic nerve fibers in the spider monkey *J Physiol* (Lond) 1960 *154* 572—580

Ingvar D H, Spectral sensitivity, as measured in cerebral visual centres *Acta physiol scand* 1959 *46* Suppl 159 1—105

Jasper H H, The ten twenty electrode system of the International Federation *Electroenceph clin Neurophysiol* 1958 *10* 371—375

Jonkman E J, The average cortical response to photic stimulation N V Drukkerij Trio s Gravenhage (Amsterdam) 160 6 1967

Jung R, Neuronale Grundlagen des Hell Dunkelsehens und der Farbwahrnehmung *Per d Ophthalm Ges* (Heidelb) 1964

Lennox Buchtal M A Single units in monkey, cercocebus torquatus atys cortex with narrow spectral responsiveness *Vision Res* 1962 2 1

Lennox M A Geniculate and cortical responses to colored light flash in cat *J Neurophysiol* 1956 *19* 271—279

Lennox M A Single fiber responses to electrical stimulation in cat s optic tract *J Neurophysiol* 1958a *21* 62—69

Lennox M A The on responses to colored flash in single optic tract fibers of cat Correlation with conduction velocity *J Neprophysiology* 1958b *21* 70—81

Lennox M A and A Madsen Cortical and retinal responses to colored light flash in anesthetized cat *J Neurophysiol* 1955 *18* 412—424

Mac Nichol E F, Retinal mechanisms of colour vision *Vision Res* 1964 4 119—133

Mac Nichol E F Jr M L Wolbarsht and H G Wagner Electrophysiological evidence for a mechanism of color vision in the goldfish In *Light and Life* ed McElroy W B and Glass H B Johns Hopkins Press Baltimore (1961)

Mac Nichol E F Jr and G Svaetichin Electric responses from the isolated retinas of fishes *Amer J Ophthal* 1958 *46* 26—39

Madsen A and M A Lennox Response to colored light flash from different areas of optic cortex and from retina in anesthetized cat *J Neurophysiol* 1955 *18* 574—582

Monnier M L organisation corticale de la vision chromatique chez l animal et chez l Homme *J Suisse Med* 1963 93 1024—1039

Monnier M and J Rozier Reponses rétiniennes et potentiels corticaux évoqués par stimulation chromatique chez l homme *J Physiol* (Paris) 1967 59 269—270

Monnier M O Watter and L Hossli Action of colour flicker stimulation on the electrical responses of retina and cortex in man *Docum ophthal* (Den Haag) 1964 *18* 207 2 0

Motokawa K K Iwama and M Ebe Retinal color processes in cats *Jap J Physiol* 1952 2 198—207

Naka K I An attempt to analyse colour reception by electrophysiology *J Physiol* (Lond) 1966 *185* 556 586

Regan D Average steady state responses to coloured stimuli *Electroenceph clin Neurophysiol* 1967 22 391

Rushton W A H The chemical basis of colour vision *New Scientist* 1961 235 3 1 377

Sanders D T and F Liske Flashing color and the electroencephalogram Neurology Minneap 1967 *17* 157 161

Shipley T R W Jones and A Fry Evoked visual potentials and human color vision *Science* 1965 *150* 1162—1163

Siegfried J B D I Tepas H G Sperling and R H Hiss Evoked brain potential correlates of psychological responses heterochromatic flicker photometry *Science* 1965 *149* 321—322

Stiles W S Color vision The approach through increment threshold sensitivity *Proc nat Acad Sci* Wash 1959 *45* 100 114

Wiesel T N and D H Hubel Spatial and chromatic interactions in the lateral geniculate body of the rhesus monkey *J Neurophysiol* 1966 29 1115 1156

Acta physiol. scand. 1969. 76. 93—105

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Effects of Epsilon Aminocaproic Acid (EACA) and Trasylol on Thromboplastin-Induced Intravascular Coagulation, Studied in Dogs with Iodine-Labelled Fibrinogen

By

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Received 6 August 1968

Abstract

NORDSTROM S and E ZETTERQVIST *Effects of epsilon aminocaproic acid (EACA) and Trasylol on thromboplastin-induced intravascular coagulation, studied in dogs with iodine labelled fibrinogen* Acta physiol scand 1969 *76* *93*—105

The elimination of iodine labelled fibrinogen was followed in 3 groups of dogs after 1 thromboplastin infusion 2 simultaneous infusions of thromboplastin and epsilon aminocaproic acid (EACA), and 3 simultaneous infusions of thromboplastin and Trasylol Platelet count fibrinogen concentration radioactivity in plasma and in fibrinogen as well as hematocrit were determined Thromboplastin caused changes of the same type as those previously obtained with thrombin indicating the development of a fibrinolytic process, secondary to intravascular coagulation The secondary changes were effectively blocked by pretreatment with EACA and Trasylol The incidence of and mortality in thromboembolism suggest some anticoagulant effect of Trasylol The antifibrinolytic activity of Trasylol is much stronger than the anticoagulant one EACA too seems to give some protection against thromboplastin induced coagulation

In previous investigations (Kowalski *et al* 1965, Nordstrom and Zetterqvist 1968) on the elimination of iodine-labelled fibrinogen after thrombin infusion in dogs, it was shown that an initial fall in radioactivity in both plasma and in thrombin *coagulable fibrinogen* was followed by *an increase in plasma radioactivity This increase* was presumed to reflect a fibrinolytic process, secondary to the thrombin induced intravascular coagulation This hypothesis was supported by the findings (Nordstrom and Zetterqvist 1968) that the secondary plasma radioactivity increase could be prevented by treatment with the antifibrinolytic drugs Trasylol and epsilon aminocaproic acid (EACA) As expected the coagulation promoting effects of thrombin were not influenced by the aforementioned drugs Independently Amris (1964) and Blomback, Blomback and Olsson (1964) found the effect of Trasylol *in vitro* to be directed not against thrombin but against an earlier phase of coagula-

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tion According to Blombäck, Blombäck and Olsson Trasylol inhibits one or several of the coagulation reactions preceding the factor V-involved step

The present investigation was made in order to study the *in vivo* effect of Trasylol and EACA on thromboplastin-induced intravascular coagulation by means of iodine-labelled fibrinogen. A preliminary report of this work has been given (Nordström and Zetterqvist 1967)

Material

Fibrinogen

Do [illegible]
no [illegible]
(1 [illegible]
mi [illegible]
used

^{131}*I labelled dog fibrinogen* was prepared essentially according to McFarlane (1956) as described for human ^{131}I fibrinogen (Blombäck *et al* 1966) On one occasion, fibrinogen was labelled with ^{125}I using the monochloride method of McFarlane (1958) The coagulability of the preparation before and after labelling was on the average 96 (range 88—99 and 93 (range 87—98 per cent, respectively The radioactivity in fibrin in per cent of that in fibrinogen was as a mean 85 (range 80—92) per cent The labelling was on the average 0 6 (range 0 2 —1 0) atoms of iodine per mole of fibrinogen The iodinated fibrinogen was stored at −30 C for 2—14 days

Other substances

Epsilon aminocaproic acid (EACA 0 1 g/ml) was the commercial product Epsikapron® of KABI Stockholm

Trasylol® a kallikrein inhibitor prepared from bovine lung containing 20 000 KIU (kallikrein inhibitor units per ml was supplied by Bayer Farma AB Stockholm.

Dog brain thromboplastin was prepared essentially according to Owren (1949 Four thromboplastin preparations were used in the experiments. The activity of each batch was calculated by comparing the dilution curve obtained by plotting log coagulation time against log concentration with a reference curve as used by Astrup (1965 The activity of each preparation was expressed in arbitrary units per ml One unit was defined as the amount of thromboplastin producing a clotting time of 18 5 sec with dog plasma assayed by the one stage prothrombin time according to Quick Nordstrom to be published)

Methods

Fibrinogen concentration fibrinolytic activity radioactivity the basic half life of the injected labelled fibrinogen platelet count and *hematocrit* were determined as described elsewhere (Nordstrom and Zetterqvist 1968

Experimental procedure

25 mongrel dogs weighing 8 17 kg were injected i v with the iodinated fibrinogen in doses giving an initial radioactivity of 40—400 / 10^{-4} µC per ml plasma The dogs were given potassium iodide one 0 5-g tablet daily from 2 3 days before the injection On the 5th or 6th day after injection of the labelled protein the dogs were anesthetized by i v injection of a 6 % pentobarbital sodium solution After 1/2 hr 3 5 to 10 ml of a thromboplastin suspension were given through a polyethylene catheter into a central vein during 10 to 21 sec

The dogs were treated as follows

Group I 8 dogs were given thromboplastin alone in doses of 1 3 to 12 [illegible] mean [illegible] arb units per kg b w

Group II 6 dogs were given thromboplastin in doses of 1 0 7 9 mean 3 9 arb units per kg b w and EACA in doses of 0 2 to 0 8 g per kg b w Half of the EACA dose was given as a single injection, immediately followed by the other half administered as a drip infusion for 45 min. The thromboplastin injection was given 15 min after the first half of the EACA dose

Group III 11 dogs were given thromboplastin in doses of 1 3 to 7 2 arb units mean 4 3 per kg b w and Trasylol in doses of 10 000 to 100 000 KIU per kg b w The Trasylol dose was administered in the same way as the EACA in group II

Results

In a previous series of dogs, the effects of thrombin on ^{131}I-labelled fibrinogen were studied (Nordstrom and Zetterqvist 1968) The general findings in that study are also valid in the present investigation Thus, the difference between the radioactivity of plasma and of thrombin coagulable fibrinogen, present already before the thromboplastin injection, is explained by the lower specific activity of fibrin than of fibrinogen in the labelled preparation This difference depends partly on the amount of radioactivity (7—8 per cent of the total label) incorporated in the fibrinopeptide B which is split off from the fibrinogen molecule during coagulation (Nordstrom *et al* 1966, Blomback and Zetterqvist 1968) Denaturation by overiodination and labelled impurities in the preparation might also have contributed Normal values for the basic half life of ^{131}I-fibrinogen in plasma and in coagulated fibrinogen were 2 0 and 1 9 days, respectively, and for plasma fibrinolytic activity in dogs 89 7 μg per ml/hr (s d 19 6) Although anesthesia and saline infusion caused some changes in the radioactivity curves, they did not markedly influence the overall results of this study

Group I Thromboplastin injection

Fig 1 shows the radioactivity curves obtained in one dog given a large dose of thromboplastin Immediately after thromboplastin injection, there was an increased rate of disappearance of radioactivity in plasma and in thrombin coagulable fibrinogen Already in the sample taken 15 min after injection, the curves for whole plasma and fibrinogen had started to diverge The radioactivity of plasma increased and reached a maximum 1 hr after injection In contrast to the plasma radioactivity that of the thrombin coagulable fibrinogen remained low The decrease in fibrinogen concentration parallelled the decrease in coagulable fibrinogen radioactivity There was also a rapid fall in the platelet count

The changes in the other dogs in this group were of the same type although with increasing thromboplastin doses the maximum value for the plasma radioactivity seemed to be reached earlier

In Table I the mean values for fibrinogen non clottable labelled fibrinogen products (NCFP) and fibrinolytic activity are given for the 5 dogs which survived 4 hrs

The fibrinolytic activity in plasma was not elevated before the thromboplastin injection Thereafter there was an increased activity with a maximum at 15 min but normalization within 2 hrs

Based on the pre infusion specific activity of the fibrinogen radioactivity, the secondary plasma radioactivity increase—representing non-clottable labelled fibrinogen products (NCFP)—can be calculated as equivalent amounts of fibrinogen Thus

TABLE I. Effects of thromboplastin injection

Mean values

	Number of dogs	Thromboplastin arb. units per kg b.w. and sec	Thromboplastin arb. units per kg b.w.	Control (before anaesth.)	Time after thromboplastin injection hrs ¼	½	1	2	4	6
Fibrinogen	3	0.23	4.2	0.50	0.29	0.29	0.26	0.28	0.28	[0.31]
g/100 ml	2	0.13	2.0	0.46	0.15	0.15	0.14	0.15	0.15	0.17
	all 5	0.19	3.4	0.48	0.23	0.23	0.21	0.23	0.23	[0.24]
Labelled non-	3	0.23	4.2	0	0.103	0.160	0.150	0.117	0.115	[0.014]
clottable fibrinogen				(0)	(53)	(75)	(66)	(54)	(44)	
products (NCFP)	2	0.13	2.0	0	0.070	0.129	0.154	0.142	0.105	0.077
g/100 ml				(0)	(24)	(42)	(50)	(47)	(36)	(26)
in % of fibrino-	all 5	0.19	3.4	0	0.090	0.148	0.151	0.127	0.111	[0.046]
gen lost				(0)	(41)	(62)	(60)	(51)	(41)	
Fibrinolytic	3	0.23	4.2	43	298	183	160	[illegible]	101	[76]
activity µg/ml/hr	2	0.13	2.0	34	122	[198]	70	45	[illegible]	34
pH 5.9	all 5	0.19	3.4	39	228	[187]	124	33	70	[55]

Values in [ ] means one sample missing

was done as previously described for fibrinogen degradation products (Nordström and Zetterqvist 1968). At the time of the "peak" the amount of NCFP ranged from 43 to 100 per cent of the fibrinogen decrease.

Of the 8 dogs, 6 died within 20 hrs. Of these, 2 were given a large dose of thromboplastin and died immediately, the other 4 died between 2 and 20 hrs after the injection. At autopsy, 5 of the 6 dogs which died during the experimental day showed occluding thromboemboli in the right side of the heart and the pulmonary arteries. Of the 2 dogs surviving the experiment and killed after one week, one had small thromboemboli, whereas in the other none could be detected.

Group II. Thromboplastin injection in EACA-treated dogs

The doses of EACA ranged from 0.2 to 0.8 g/kg b.w. The radioactivity curves initially showed about the same slope as in the dogs given thromboplastin only. Thereafter in the 2 dogs given the largest dose of EACA (0.8 g) only a weak secondary increase occurred in the plasma radioactivity (Fig. 2), whereas in the other 4 the curves mainly followed the same course as those in the thromboplastin group.

The fibrinogen concentration changed initially in roughly the same way as with thromboplastin alone (Table II). The decrease in platelet count was somewhat smaller.

Results in the 5 dogs which survived 4 hrs are presented in Table II. In . dogs

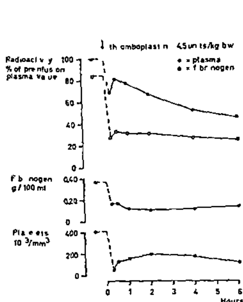


Fig 1

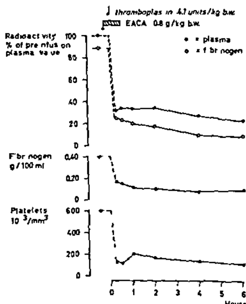


Fig 2

Fig 1 Injection of *thromboplastin* 5 days after i v injection of ^{131}I fibrinogen in dog no 595 Plasma radioactivity before experiment 6.0×10^{3} μC/ml Plasma and fibrinogen radioactivity, fibrinogen concentration and platelet count

Fig 2 Injection of *thromboplastin* during infusion of a *large dose of EACA* in dog no 567 Plasma radioactivity before experiment 5.2×10^{3} μC/ml

in which fibrinolysis was determined, no significantly increased activity was demonstrated during EACA treatment The maximum for calculated NCFP in the dogs treated with large doses of EACA appeared 2 to 4 hrs after thromboplastin injection and was estimated at 8 and 33 per cent, respectively, of the fibrinogen decrease

The 2 dogs given the largest EACA dose died from occluding thromboemboli within 1 and 4 days Of the 4 surviving dogs killed after one week, 3 had small to moderate thromboemboli in the right ventricle, whereas in one no emboli were found

Group III Thromboplastin injection in Trasylol treated dogs

Trasylol was given before and after the thromboplastin injection in doses from 10 000 to 100 000 units per kg b w The radioactivity curves initially ran as in the thromboplastin group In the dogs given a medium dose (50,000 units), the plasma radioactivity increase was delayed (Fig 3) and in those given a small dose, a peak appeared at the same time as in the thromboplastin group In the dogs given the largest dose of Trasylol the plasma radioactivity increased somewhat from 4 hrs and onwards but no peak was noted (Fig 4) The initial drop in fibrinogen concentration after thromboplastin injection was slightly smaller than in the other groups (Table IV) The platelet count fell to the same level as in EACA treated dogs

TABLE II Effects of intravenous injections of EACA and thromboplastin

Mean values

	Number of dogs	EACA g/kg b w	Thromboplastin arb units per kg b w	Thromboplastin arb units per kg b w and sec	Control (before anesth)	Time after thromboplastin injection hrs ¼	½	1	2	4	6
Fibrinogen g/100 ml	3	0.2	0.22	3.6	0.44	0.28	0.26	0.25	0.25	0.29	[0.24]
	2	0.8	0.14	2.6	0.47	0.18	0.15	0.12	0.05	0.05	0.05
	all 5	0.44	0.19	3.2	0.45	0.24	0.22	0.20	0.17	0.19	[0.15]
Labelled non clottable fibrinogen products (NCFP) g 100 ml in ( ) % of fibrinogen loss	3	0.2	0.22	3.6	0 (0)	0.031 (22)	0.067 (37)	0.105 (69)	0.096 (60)	0.046 (27)	[0.059]
	2	0.8	0.14	2.6	0 (0)	0 (0)	0.005 (1.3)	0.018 (5)	0.071 (15)	0.096 (20)	0.086 (17)
	all 5	0.44	0.19	3.2	0 (0)	0.019 (9)	0.042 (18)	0.070 (27)	0.086 (30)	0.066 (23)	[0.073]
Fibrinolytic activity µg/ml hr pH 5.9	2	0.2	0.13	2.7	119	53	73	41	[102]	[109]	21

Values in [ ] means one sample missing

One dog given 50 000 units of Trasylol had before the experiment a high fibrinogen concentration (0.68 g/100 ml) and also high fibrinolytic activity (474 µg per ml/hr) in the circulating blood Despite a fairly large thromboplastin dose there was no decrease in the fibrinogen concentration and a moderate fall in the platelet count of 35—40 per cent In this dog the fibrinolytic activity decreased but did not disappear completely

Increased fibrinolytic activity was found 2 to 6 hrs after thromboplastin injection in one dog given 10 000 KIU and at 6 hrs in one dog given 100 000 KIU Trasylol per kg b w The maximum for NCFP appeared successively later with rising Trasylol doses In the dogs given 100 000 units of Trasylol the highest amounts of NCFP (14 and 54 per cent respectively of the fibrinogen decrease were noted as late as 6 hrs after the thromboplastin injection (Table III)

Of the 11 dogs in this group 3 died immediately after the thromboplastin injection. Another 3 dogs died within the first 20 hrs Five of these dogs had massive thromboemboli in the right side of the heart and pulmonary arteries Autopsy was not performed in one dog The 5 surviving dogs were killed one week after the experiment One of them had small thromboemboli in the tricuspid valve the other 4 showed no signs of thrombi

TABLE III Effects of intravenous injections of Trasylol and thromboplastin

Mean values

	Number of dogs	Trasylol KIU/kg b w	Thromboplastin arb units per kg b w and sec	Thromboplastin arb units per kg b w	Control (before anesth)	Time after thromboplastin injection hrs ¼	½	1	2	4	6
Fibrinogen	3	10,000	0 21	3 9	0 39	0 19	0 18	0 18	0 17	0 17	0 19
g/100 ml	2	100,000	0 13	2 5	0 43	0 29	0 25	0 23	0 25	0 25	0 26
	all 5	46,000	0 18	3 4	0 40	0 23	0 21	0 20	0 20	0 20	0 22
Labelled non-clottable fibrinogen	3	10,000	0 21	3 9	0	0 011	0 029	0 044	0 050	0 033	0 022
					(0)	(6)	(16)	(24)	(24)	(15)	(11)
products (NCFP)	2	100,000	0 13	2 5	0	0 010	0 013	0 013	0 018	0 036	0 069
g/100 ml					(0)	(10)	(11)	(7)	(10)	(20)	(34)
in ( ) % of	all 5	46,000	0 18	3 4	0	0 011	0 022	0,032	0 037	0 034	0 041
fibrinogen loss					(0)	(8)	(14)	(17)	(19)	(17)	(20)
Fibrinolytic	2	10,000	0 19	3 6	92	37	9	10	91	163	167
activity µg/ml/hr	2	100,000	0 13	2 5	36	72	55	49	78	46	121
pH 5 9	all 4	55,000	0 16	3 1	64	54	32	29	84	104	144

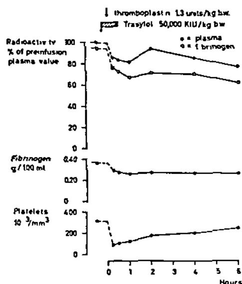


Fig 3

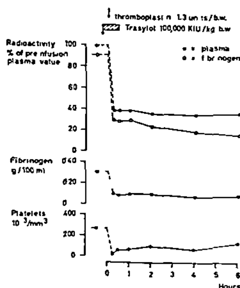


Fig 4

FIG 3 Injection of *thromboplastin* during infusion of a *medium dose of Trasylol* in dog no 446 Plasma radioactivity before experiment 10.0×10^{3} µC/ml

FIG 4 Injection of *thromboplastin* during infusion of a *large dose of Trasylol* in dog no 410 Plasma radioactivity before experiment 7.7×10^{3} µC/ml

TABLE IV. Early effects (15 min) of thromboplastin injection in dogs untreated (I) and pretreated with EACA (II) and Trasylol (III)

Mean values in per cent of preinjection values

Group	Number of dogs	Thromboplastin arb. units per		Fibrinogen radio-activity	Fibrinogen conc.	Platelet count
		kg b.w. and sec	kg b.w.			
I	6	0.19	3.5	45	55	17
II	6	0.22	3.9	50	52	30
III	8	0.21	3.9	63	62	32

Comparison between the groups

The mean values for the doses of thromboplastin in units per kg b.w. and sec were 0.31 in group I, 0.22 in group II, and 0.26 in group III. Mean values for the total amount of thromboplastin in arbitrary units per kg b.w. were 4.8, 3.9 and 4.3, respectively, in the corresponding groups. One dog in group I was given a very large dose which explains the higher mean values in this group. Two dogs given thromboplastin ne and 3 Trasylol treated dogs died immediately after thromboplastin injection.

The *early* effects of the thromboplastin injections in those dogs which survived 15 min are illustrated in Table IV. The decrease of fibrinogen radioactivity and concentration seemed to be smaller in the Trasylol group than in the other groups. The fall in platelet count was equally influenced by EACA and Trasylol.

For evaluation of the *late* effects, a comparison was made between dogs surviving 4 hrs. In addition to those animals which died within 4 hrs, the following dogs were excluded from the comparison (Tables I—III and V).

Group II: one dog given 0.4 g EACA per kg b.w., because samples could not be obtained after 2 and 4 hrs.

Group III: two dogs given 50 000 KIU Trasylol per kg b.w., one because samples could not be obtained after 4 hrs, the other because of the high pre-experimental fibrinolytic activity in the circulating blood, as mentioned above.

To obtain corresponding thromboplastin doses and equal numbers of dogs in the three groups, another dog—given 50 000 KIU Trasylol per kg b.w.—was excluded. This dog is presented in Fig. 3.

The groups were thus reduced to 5 dogs in each. The thromboplastin doses are comparable. In Tables I—III fibrinogen concentration, fibrinolytic activity and NCFP are presented for each group.

The calculated amount of NCFP was higher and the maximum was reached earlier after large thromboplastin doses than after small (Table I). With increasing doses of

TABLE V Effects of thromboplastin injection in dogs untreated (I) and pretreated with EACA (II) and Trasylol (III)

Mean values in per cent of preinjection values

	Group	Number of dogs	Time after thromboplastin injection hrs 1/4	4
Fibrinogen radio-activity	I	5	43	40
	II	5	54	37
	III	5	50	40
Fibrinogen concentration	I	5	47	46
	II	5	54	44
	III	5	49	43
Platelet count	I	5	15	48
	II	5	31	55
	III	5	23	44

EACA and Trasylol the maximum of NCFP became successively smaller and appeared later

As can be seen in Table V, the initial decrease in fibrinogen radioactivity and *platelet count was somewhat smaller in EACA and Trasylol treated dogs than in* those given thromboplastin alone During the following 4 hrs, fibrinogen radioactivity and concentration remained on the same level in group I but continued to fall in the other groups The platelets seemed to return faster in the untreated group

During the first 24 hrs, the mortality was 6 of 8 in group I 1 of 6 in group II, and 6 of 11 in group III

Macroscopical thromboemboli in the right side of the heart and the pulmonary arteries were found in 7 of 8 dogs in group I, in 5 of 6 in group II, and in 6 of 10 in group III

The hematocrit fell in groups I and III by 10—15 per cent during the first 1/2 hr In the EACA treated dogs however, the hematocrit rose to a value about 20 per cent above the initial level All plasma values were therefore corrected for changes in hematocrit

Discussion

Intravascular coagulation can be induced experimentally in several ways Thrombin or thromboplastin has been used for this purpose by many investigators (Wooldridge 1886 Quick *et al* 1959 and others) In a previous study we tested the effects of thrombin on the elimination of fibrinogen in dogs using ^{131}I fibrinogen as a tracer (Nordstrom and Zetterqvist 1968)

Thromboplastin compared to thrombin has the disadvantage of being more cult to standardize with respect to the coagulation promoting effect Like Astrup used a reference curve for comparison between the activity of different throm plastin preparations The reaction to the injection also seems to be influence individual variations in the general condition and sensitivity of the animals (I 1967 Nordstrom unpublished data)

In general the effects obtained with thromboplastin seemed to be similar to t produced by thrombin In dogs infused with thrombin during 1 hr the initial pl radioactivity decrease was followed by an increase with a maximum 2 hrs after start of the infusion Although in the experiments with thrombin, no significantl creased fibrinolytic activity could be demonstrated in the circulating blood there evidence that this subsequent plasma radioactivity increase could have been ca by a fibrinolytic process probably induced secondarily to intravascular coagula This is in accordance with the suggestions of Kowalski *et al* 1965

The thromboplastin doses were given within half a minute and the maxi reactions occurred earlier and more dramatically than in the dogs given thrombin contrast to the findings in the thrombin experiments an elevated fibrinolytic act was recorded in the circulating blood Because of the shorter injection time thromboplastin the coagulation trauma was probably more intense This might explain why a fibrinolytic process could be more intensely evoked and there demonstrable in the circulating blood By treatment with fibrinolysis inhibito EACA and Trasylol—it was possible in this investigation as well to delay or alr hibit the secondary plasma radioactivity increase and to prevent an increas fibrinolytic activity in the circulating blood during the first hours This supports hypothesis that the secondary plasma radioactivity increase is caused by a fibrinol process

With increasing doses of thromboplastin higher fibrinolytic activity and prop tionally larger amounts of NCFP appeared in the circulation indicating an intim relation between the intensity of the coagulation process and the fibrinolytic respo By using EACA and Trasylol a correlation was obtained between the dose of antifibrinolytic drug and the effect on the induced fibrinolytic activity With 0 8 g EACA or 100 000 KIU of Trasylol per kg b w administered intravenously withi hr a pronounced inhibition of the fibrinolytic process was exerted for at least 4 The effect of 100 000 KIU of Trasylol was greater than that of 0 2 g of EAC which implies that on a molar basis Trasylol was much more effective than EAC This is in accordance with the observations of Dubber *et al* (1968) who found molar potency of Trasylol as an inhibitor of fibrinolysis in an *in vitro* stand plasma clot lysis system to be 1 000 times that of EACA

It must be pointed out that the radioactivity curves given in the figures as w as the other data calculated from the radioactivity values are influenced methodological and experimental measures Thus the fibrinogen radioactivity val do in fact represent the radioactivity in fibrin since the samples were measu after clotting When dog fibrinogen is converted to fibrin around 11 per cent of

total fibrinogen label is lost (Regoeczi and Walton 1967) and 7—8 per cent of the latter has been found in the isolated fibrinopeptide B (Blomback and Zetterqvist 1968) Furthermore it has recently been demonstrated in dogs that indwelling polyethylene catheters for 8 hrs may double the turnover rate of iodine labelled fibrinogen (Zetterqvist Carlson and Liljedahl 1968) Loss of radioactivity by sampling will also influence the estimations of radioactivity decrease and NCFP

The calculations are made without taking into account the basic decay the different amounts of released fibrinopeptide B and blood sampling Such corrections will however tend to increase the quantities of NCFP by less than 10 per cent rather than to decrease them It seems probable that the radioactivity changes following coagulation indwelling catheters and blood sampling all have an influence of about the same degree in all dogs We therefore believe that a comparison between the groups can be made without regard to these influences

As far as the hematocrit is concerned there was a tendency to decreasing values in most of our experiments on induced intravascular coagulation (Nordstrom unpublished data Nordstrom and Zetterqvist 1968) However in EACA treated dogs the hematocrit increased immediately after thromboplastin injection. This is in accordance with the report of Cummings and Welter (1966) who found intravenous administration of large amounts of aminocaproic acid to evoke a syndrome resembling deshydremic shock with decreased total peripheral resistance oedema and hemoconcentration They suggested that the drug may have a direct effect on the cardiovascular system in addition to a catecholamine depleting action. Under these circumstances the difference in hematocrit changes between the groups is probably due to differences in plasma volume A more correct comparison between the groups would therefore be obtained when based on values corrected for hematocrit

It has been claimed that Trasylol *in vitro* inhibits thromboplastin (Amris 1964 Blomback Blomback and Olsson 1964) In view of this statement, an evaluation of the *in vivo* effects of Trasylol on the early changes after thromboplastin injection seemed to be of interest When all the dogs surviving 15 min were considered Trasylol seemed to inhibit the coagulation process to some degree (Table IV) However in those dogs surviving 4 hrs (Table I—III and V) only small differences were observed between the groups In EACA and Trasylol treated dogs a slight retardation of the thromboplastin effect seemed to occur

With respect to the effect of Trasylol it has been shown both in this investigation and earlier ones (Nordstrom Olsson and Blomback 1966 Mammen and Poucho 1966) that the incidence of and mortality in thromboembolism in thromboplastin injected dogs were lower if the animals were pretreated with Trasylol In dogs infused with thrombin on the other hand the mortality was higher when the dogs received Trasylol (Nordstrom and Zetterqvist 1968 This is not surprising since Trasylol is an effective antifibrinolytic drug and has no influence on the thrombin time (Amris 1964 Blomback Blomback and Olsson 1964 Godal and Theodor 1965 Andersson Nilsson and Hedner 1968) This difference between the mortality in the

dogs given thromboplastin-Trasylol and those given thrombin-Trasylol points to some antithromboplastin effect of Trasylol It does not, however, rule out the mechanism suggested by Mammen and Poucho, *i e*, that the action of Trasylol is directed against the final phase, causing an impaired cross linking of the polymerized fibrin monomers Mammen and Poucho explained their results on the basis of disturbed clot retraction and decreased fibrin stabilizing factor level (F XIII) in Trasylol treated dogs *In vivo*, Trasylol might have a dual effect on the coagulation process

EACA prolongs the thrombin time, and this effect is accentuated by the presence of fibrinogen degradation products (Godal and Theodor 1965) Godal and Helle (1963) found that *fibrinogen degradation products markedly inhibit polymerization* of fibrin As proposed by Abildgaard (1964), the prolonged thrombin time is most likely due to impaired polymerization of fibrin Such an effect on the polymerization process of EACA might explain the low mortality in dogs given EACA before administration of thromboplastin and thrombin However when very high doses of EACA are given, fibrinolysis will also be blocked, and the clots formed—although soft and not crosslinked—will still not be lysed This might explain the death of these 2 dogs given the largest EACA dose

The number of animals in this investigation is too small to permit definite conclusions about the *in vivo* effect of Trasylol The mortality in and incidence of thromboembolism nevertheless suggest that it has some anticoagulant effect The antifibrinolytic activity of Trasylol is much stronger than its anticoagulant one EACA also seems to give some protection against thrombin- and thromboplastin induced coagulation probably by impairing the polymerization of fibrin monomers

This investigation was supported by grants from the Swedish Medical Research Council (grant no K67 19X 520 03) the Swedish National Association against Heart and Chest Disease and from Bayer Farma AB Stockholm

References

ABILDGAARD U Acceleration of fibrin polymerization by acetate and other low molecular ions *Scand J clin Lab Invest* 1964 *16* 521—528

AMRIS C J Inhibition of fibrinolytic and thromboplastic activity by Trasylol *Abstr Xth Congr Int Soc Haemat* 1964 G 72 (*Scand J Haemat* 1966 *3* 19—32

ANDERSSON L I M NILSSON and L HEDNER Experimental and clinical studies on the antifibrinolytic and presumed antithrombotic effect of a kallikrein inhibitor (Trasylol *Scand J Urol Nephrol* 1968 *1* 11—18

ASTRUP T Assay and content of tissue thromboplastin in different organs *Thrombos Diathes haemorrh* (Stuttgart) 1965 *16* 401—416

BLOMBÄCK B and M BLOMBACK Purification of human and bovine fibrinogen *Arkiv Kemi* 1956 *10* 415—443

BLOMBÄCK B M BLOMBACK and E HOLMBERG A new method for fractionation of proteins *Acta chem scand* 1966 *20* 2317 2319

BLOMBÄCK, B M BLOMBACK and P OLSSON Action of a proteolytic enzyme inhibitor on blood coagulation *in vitro* Pers comm 1964 (*Neue Aspekte der Trasylol Therapie* F K Schattauer Verlag Stuttgart 1966 pp 33—43)

BLOMBÄCK B, L A CARLSON S FRANZEN and E ZETTERQVIST Turnover of ^{131}I labelled fibrinogen in man *Acta med scand* 1966 *179* 557—574

BLOMBÄCK B and E ZETTERQVIST Incorporation of ^{131}I into dog fibrinopeptides 1968 To be published

CUMMINGS J R and A N WELTER, Cardiovascular studies on aminocaproic acid *Toxicol appl Pharmacol* 1966 *9* 57—69

DUBBER, A H C, G P MCNICOL, D UTTLEY and A S DOUGLAS, *In vitro* and *in vivo* studies with Trasylol, an anticoagulant and a fibrinolytic inhibitor *Brit J Haemat* 1968 *14* 31—49

GODAL, H C and I HELLE, The influence of fibrinogenolytic and fibrinolytic split products on the last stage of coagulation *Scand J clin Lab Invest* 1963 *15* 327—330

GODAL, H C and I THEODOR, *In vitro* inhibition of streptokinase induced proteolysis by Trasylol and by epsilon aminocaproic acid (EACA) *Scand J clin Lab Invest* 1965 *84* 199—208

HJORTH, P F, Disc at the *Grisslehamn Conf on Intravascular Coagulation*, May 1967

KOWALSKI E, A Z BUDZYŃSKI, M KOPEĆ, Z S LATALLO, B LIPINSKI and Z WEGRZYNOWICZ, Circulating fibrinogen degradation products (FDP) in dog blood after intravenous thrombin infusion, *Thrombos Diathes haemorrh* (Stuttg) 1965 *13* 12—24

MAMMEN, E F and G G POLSHO The effect of Trasylol on the intravascular coagulation of blood in dogs (Disc at the N Y *Acad Sci Conf on Proteinase Inhibitors* Sept 1966) N Y Acad. Sci 1968 *146* 713—714

MCFARLANE, A S, Labelling of plasma proteins with radioactive iodine *Biochem J* 1956 *62* 135—143

MCFARLANE, A S, Efficient trace-labelling of proteins with iodine *Nature* (Lond 1958 *182* 53

NORDSTRÖM, S Inhibition of blood coagulation by Trasylol (To be published)

NORDSTROM, S, B BLOMBÄCK, M BLOMBACK, P OLSSON and E ZETTERQVIST, Experimental investigations on the antithromboplastic and antifibrinolytic activity of Trasylol (Lect at the N Y *Acad Sci Conf on Proteinase Inhibitors* Sept 1966) *Ann N Y Acad Sci* 1968 *146* 701—711

[illegible]

OWREN P A quantitative one stage method for the assay of prothrombin *Scand J clin Lab Invest* 1949 *1* 81—83

QUICK A J, C V HUSSEY J HARRIS and K PETERS, Occult intravascular clotting by means of intravenous injection of thrombin *Amer J Physiol* (Lond) 1959 *197* 791—794

REGOECZI, E and P L WALTON Effects of clotting on the label in iodinated fibrinogen in different species *Thrombos Diathes haemorrh* (Stuttg) 1967 *17* 237—246

WOOLDRIDGE, L C Über intravasculare Gerinnungen *Archiv fur Anatomie and Physiologie* 1886 *10* 397—399

ZETTERQVIST E L A CARLSON and S O LILJEDAHL Influence of indwelling polyethylene catheters on the elimination of [125]I labelled fibrinogen in dogs. Lect. at the *International Conference on Microcirculation* Gothenburg June 1968

Acta physiol. scand. 1969. 76. 106—120

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Histochemical Studies on the Metabolism of L-DOPA[1] and Some Related Substances in the Exocrine Pancreas

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Received 7 August 1968

Abstract

Alm P, B Ehinger and B Falck *Histochemical studies on the metabolism of L-DOPA and some related substances in the exocrine pancreas* Acta physiol scand 1969 76 106—120

The turnover of L DOPA and some related substances was studied with the fluorescence method of Falck and Hillarp All the amino acids injected were easily taken up by the pancreas This however was not the case with the corresponding amines Only after L DOPA and DA administration fluorescent granules could be seen in the apical zones of the exocrine cells These granules were found to be identical with the zymogen granules Decarboxylase inhibition prevented and MAO and COMT inhibition prolonged the appearance of the fluorescent granules This indicates that the fluorescent products stored in the zymogen granules is dopamine rather than DOPA

The pancreas intensively synthetizes and secretes proteins Compared with many other organs it has a large capacity for taking up amino acids from the blood (see e g Busch et al 1959 Hansson 1959)

It takes up not only those that are essential for the protein synthesis but also a number of other amino acids and analogues of amino acids selenomethionine (Blau and Manske 1961 Hansson and Jacobsson 1966) parafluorophenylalanine (Garzo, Hansson and Ullberg 1962) 1-amino-cyclopentane carboxylic acid (Berlinguet Begin and Babineau 1962) L-DOPA (Rosell Sedvall and Ullberg 1963 Cegrell Falck and Hellman 1964) glutamine (Cassano and Hansson 1965) and 5 HTP (Ritzen Hammarstrom and Ullberg 1965 Gershon and Ross 1966) This uptake perhaps represents a specific mechanism for the metabolism of these amino acids but it could also be a part of the general mechanism behind the protein synthesis Neither the morphological nor the quantitative information required to separate these two

[1] The following abbreviations have been used in this article DOPA = 3 4-dihydroxyphenylalanine DA = dopamine NA = noradrenaline A = adrenaline 5 HTP = 5 hydroxytryptophan 5-HT = 5 hydroxytryptamine MAO = monoamine oxidase COMT = catechol O-methyl transferase

points, however, is at hand It was consequently to interest to study the uptake, storage, and elimination of L-DOPA in the pancreas, which can easily be done with the very sensitive and specific fluorescence method of Falck and Hillarp Preliminary studies with L-DOPA (Alm, Ehinger and Falck 1967) has revealed new aspects on the metabolism of this substance The details concerning this turnover are described here

Material and methods

Albino mice and albino rats of both sexes (weighing about 20—25 g and 180—250 g) were used (Anticimex, Sweden) All the animals were given Standard pellets (Teknosan Sweden) and water *ad libitum* The mice were injected with the various drugs in one of the lateral tail veins after dilatation with hot water (50° C) The time for the administration was about 20—30 sec The rats were lightly anesthetized with ether, and the drugs were injected into the major saphenic vein The injection procedure took about 2 min and after this the animals woke up immediately They were sacrified by a blow on the neck at various times after the injections whereupon the pancreas was rapidly dissected out frozen in liquid nitrogen-cooled propane freeze dried treated with formaldehyde of standardized humidity (Falck and Owman 1965 Hamberger 1967), embedded in paraffin wax in vacuo sectioned and studied in a fluorescence microscope (for details see *e g* Falck and Owman 1965)

Drugs

The following substances dissolved in 0 9 % saline were injected L-DOPA (Sigma Chem Corp), D DOPA (Fluka AG), L-α methyl DOPA (Dumex Ltd), DA (Hoffman La Roche), DL-m tyrosine (Sigma Chem Corp) and DL-α methyl m tyrosine (Regis Chem. Corp)

β thujaplicin (4 isopropyltropolone Fluka AG), dissolved in 0 9 % saline was given i p (200 mg/kg) 1 hr before the injection of L-DOPA or DA NSD 1015 (m hydroxybenzyl hydrazine Smith and Nephew through Ferrosan AB Sweden), dissolved in 0 9 % saline, was given (100 mg/kg) 1 hr before the injection of L-DOPA

Nialamide solution (Niamid Pfizer Ltd) was prepared as described by Bertler Falck and Owman 1964 and injected (100 mg/kg) 3 hrs before the injection of L-DOPA or DA

Microscopy

Microscopy was performed according to Falck and Owman (1965) After the injection of DOPA DOPA analogues or DA a greenish fluorescence appeared in the exocrine pancreatic cells (see *Results* This fluorescence fulfilled the criteria formulated for the identification of

to as specific fluorescence in

1967) The fluorescence picture

localization of the fluores ence

not be localized to any cellular

structure it was considered diffuse The intensity of the fluorescence was judged subjectively 0=absence of fluorescence 1=weak 2=moderate 3=strong 4=very strong This was to make clear very simply the changes in intensity of the subjectively estimated observations It is to be understood that fluorescence intensity is not always regularly proportioned to the amine concentration (see Ritzen 1967)

Results

Normal appearance

The pancreas of normal rats and mice shows specific fluorescence only in adrenergic nerves enterochromaffin cells and small intensely fluorescent cells The adrenergic nerves follow the vessels to some extent even those of capillary size (Alm *et al* 1967) The exocrine parenchyma does not seem to be innervated by adrenergic nerves nor has it normally any specific fluorescence

The enterochromaffin cells are pointed and flask like with the yellow fluorescence characteristics for 5 HT They lie scattered in the epithelium of the largest pancreatic ducts (*cf* review by Vialli 1965



Fig 1 Rat pancreas 40 min after the injection of L-DOPA (40 mg/kg) Specific fluorescence *is localized to the apical parts of the exocrine cells Fluorescence micrograph 440 ×*

The small intensely fluorescent cells are generally localized to the pancreatic ganglia but can to a lesser extent also be found in the connective tissue Microspectrofluorimetric analyses have shown that these cells contain a primary catechol amine (Alm *et al* 1967) This together with their morphology the intensity of the fluorescence and their distribution places them in the same category as the SIF cells *previously described in and at sympathetic ganglia (Norberg Ritzen* and Ungerstedt 1966) and now known to contain DA (Bjorklund Cegrell Falck Ritzen and Rosengren to be published)

Effect of drugs

The differences between mice and rats were as a rule small (*cf* the tables and deserve comments only in a few cases After the injection of L- or D DOPA the pancreatic acinar cells attained a strong specific fluorescence In a pilot time-dose response study *L-DOPA was given i v 10 20 40 60 and 100 mg/kg and the mice* were killed after 5 15 30 and 60 min Up to 30 min after the injection the specific fluorescence was intense and diffusely distributed throughout the acinar cells but in the apical part of the cells a sparse number of coarse fluorescent granules could sometimes be seen. Little or no specific fluorescent occurred in the connective tissue



Fig 2a Rat pancreas 40 min after the injection of L-DOPA (100 mg/kg) Zymogen granules are stored in the apical parts of the exocrine cells around the acinar lumen There is close correspondance between a zymogen granulum and a coarse fluorescent granulum (compare with Fig 2b Phase-contrast micrograph, 1500 ×

Fig 2b Fluorescence micrograph same area and magnification as Fig 2a With this dose of L-DOPA however, all zymogen granules became fluorescent though varying in intensity

60 min after the injection the specific fluorescence occurred only in the apical regions of the acinar cells and in the form of densely aggregated coarse granules (Fig 1 and 2 The minimum dose that gave a clear appearance of the granules was 40 mg/kg (although granules could be seen at lower dose levels) In a preliminary experiment when L-DOPA was given to rats in a dose of 40 mg/kg nearly the same morphological picture and fluorescence intensity as that of mice was obtained at the different times after the injections Thus this dose was chosen for the further study of the effect of various drugs on the metabolism of L-DOPA in the two species

In the continued series L-DOPA (40 mg/kg) was injected and the animals were killed after 10 40 60, 90, and 180 min (see Table I and III) A strong specific fluorescence appeared in the pancreatic acinar cells already after 10 min. The fluorescence was mainly diffuse although fluorescent granules could sometimes be seen At 40—60 min after the injection however the specific fluorescence was mainly confined to the apical regions of the acinar cells (Fig 1 and 2) there being little or no fluorescence in the basal 2/3 of the cells The fluorescence had all the characteristics of a primary catecholamine and appeared in densely aggregated coarse granules This type of fluorescence is in the following referred to as 'granular As

TABLE I Mouse

Time after the last injection (minutes)		10	40	60	90	120	180	240	300
Drugs									
L-DOPA	D	2	1/2	1/2	0		0		
40 mg/kg	G	0	3	2	0		0		
	N	6	7	8	6		6		
D DOPA	D	3	2 1/2	2	2		1		
40 mg/kg	G	0	0	0	0		0		
	N	6	6	6	6		6		
L-α-methyl-DOPA	D	2	2	2		1	1/2	0	
40 mg/kg	G	0	0	0		0	0	0	
	N	4	4	4		4	4	4	
DL-m-tyrosine	D		3	2	2		1/2	1	0
200 mg/kg	G		0	0	0		0	0	0
	N		2	2	2		2	2	2
DL-m-tyrosine	D	2	1	0	0		0		
40 mg/kg	G	0	0	0	0	0	0		
	N	3	3	3	3		3		
DL-α methyl-	D	2	1	1	0		0		
m tyrosine	G	0	0	0	0		0		
40 mg kg	N	3	3	3	3		3		

D = diffuse fluorescence
G = granular fluorescence
N = number of animals used
h = hour
a = acini
1/6 = in one animal out of six
2/5 = in two animals out of five etc

seen under phase contrast optics the fluorescent granules were identical with the zymogen granules (see Fig 2 a and b), although at times only a certain proportion of these displayed fluorescence 90 min after the injection of L-DOPA all specific fluorescence had disappeared in the mice In rats the fluorescent apical granules at times remained somewhat longer but had all disappeared after 180 min

After the injection of D DOPA (40 mg/kg) a diffuse fluorescence appeared all over the pancreatic acinar cells in the same way as with L-DOPA However "granular" fluorescence never appeared Instead the fluorescence stayed diffuse and gradually disappeared thus it remained longer than with L-DOPA and in mice it had not completely disappeared even at 180 min (see Table I and II)

The injection of L-α-methyl-DOPA (40 mg/kg) DL α-methyl-m-tyrosine (40 mg/kg) and DL-m-tyrosine (40 and 200 mg/kg) resulted in essentially the same diffuse fluorescence as with D-DOPA After approximately 3 hrs the fluorescence had



Fig 3 Rat pancreas 10 min after the injection of DA (40 mg/kg) Fluorescent zymogen granules with strong fluorescence is seen only in a few acini (compare with Fig 1) In some acini there are zymogen granules with a very weak fluorescence Fluorescence micrograph, 288 ×

disappeared (see Table I and III) In the case of the 3 hydroxylated derivatives the formaldehyde induced fluorescence was more yellowish than that of *e g* DOPA

DA (40 mg/kg) was taken up by the exocrine pancreas of both mice and rats although to a considerably lesser extent than both D- and L-DOPA (see Table II and IV) Ten minutes after the injection, the exocrine pancreas in both mice and rats displayed only weak specific fluorescence in the cytoplasm, which should be compared with the strong cytoplasmic fluorescence noted ten minutes after the administration of either of the two DOPA isomers The intensity of the diffuse fluorescence gradually disappeared (see Table II and IV) Fluorescent apical granules appeared *in a few* cases and then only in restricted numbers and a *few* acini (Fig 3)

After a higher dose of DA (100 mg/kg) the diffuse cytoplasmic fluorescence was still low (see Table II and IV) In mice the number of acini containing fluorescent apical granules, however was increased as well as the number of granules in the individual acini compared with the lower dose Moreover the granular fluorescence generally appeared earlier than it did after the injection of L DOPA (see Table II) Nevertheless the uneven distribution of the granules among the acini was evident in

TABLE IV Rat

Time (minutes) after the last injection		10	40	60	90	180	
Drugs							
							(I)
Dopamine 40 mg/kg	D	1/2	1/2	0	1/2	0	G in a few a
		(I)	(I)				
	G	3	2 1/2, in 1/3	0	0	0	
	N	3	3	3	3	3	
Dopamine 100 mg/kg	D	1		0			(II) in 2/3 G in a few a, in 1/3 G in 40 % of a
				(I)			
	G	0		3			
	N	3		3			
Nialamide 3 hrs + dopamine 40 mg/kg	D	1/2	1/2	1/2			(III) in 2/3 G in 70 % of a, in 1/3 G in a few a
		(I)	(II)	(III)			
	G	3 in 1/3	3	3			
	N	3	3	3			
β-thujaplicin 1 hr +	D	0	0	0			
		(I)	(II)	(III)			
dopamine 40 mg kg	G	3 in 1/3	3	3			
	N	3	3	3			
Nialamide 3 hrs +	D	1/2					
		(I)					
β thujaplicin 1 hr +	G	3, in 1/3					
dopamine 40 mg kg	N	3					

lacking granules. After 240 and 300 min apical fluorescent granules were found only in scattered acini. Thereafter the fluorescence gradually disappeared (see Table VI and VII). *A repeated injection of NSD 1015 (in rats 2 hrs and in mice 3 hrs after* the first NSD 1015 injection) completely inhibited the appearance of granular fluorescence after the L-DOPA injection. Instead the fluorescence remained diffuse though decreasing in intensity throughout the experiment (see Table V).

Discussion

The histochemical demonstration of certain biogenic monoamines (such as DA, NA, A and 5 HT) and some related substances (such as DOPA and 5 HTP) depends upon the ability *of these substances to form intensively fluorescent products when ex-*

TABLE V

Time after L-DOPA injection (hrs)		2	3	4	5	6	
Drugs							
NSD 1015 1 hr (×)	D	3	3	2			(×) rat
+ L-DOPA 40 mg/kg	G	0	0	0			
1 hr + NSD 1015	N	4	4	4			
NSD 1015 1 hr (××)	*D*		2	1 1/2	1 1/2	1	(××) mouse
+ L-DOPA 40 mg/kg	G		0	0	0	0	
2 hrs + NSD 1015	N		2	2	2	2	

TABLE VI Mouse

Time (hrs) after the last injection		30	60	120	180	240	300	360	420
Drugs									
Nialamide 3 hrs	D	3 1/2	0	0	0	0	0	0	0
+ L-DOPA 40 mg/kg	G	0	3	3	2	1, in 2/4	0	2, in 1/4	0
	N	4	4	4	4	4	4	4	4
NSD 1015 1 hr	D	3	3	3	3	0	0	0	0
+	G	0	0	0	0	3	2	1	0
L-DOPA 40 mg/kg	N	4	4	4	4	4	4	4	4
β thujaplicin 1 hr	D	1 1/2	0	0	0	0			
+	G	3	2 1/2	3	3	3			
L-DOPA 40 mg/kg	N	5	10	5	5	3			

posed to formaldehyde under certain well defined conditions. The fluorescent *products are easily recognized and localized on the cellular level under the fluo-*rescence microscope. Extensive studies on the chemical background, the specificity, and the sensitivity of the procedure have proved its reliability provided that proper precautions are taken. Specific fluorescence appears precisely where certain catecholamine or indolamine derivatives occur (Falck, Hillarp, Thieme and Torp 1962, Falck 1962; for reviews see Falck and Owman 1965, Corrodi and Jonsson 1967, Norberg 1967, Ritzen 1967).

In the present experiments

TABLE VII Rat

Time (hrs) after the last inject on		30	60	120	180	240	300	360	420	
Drugs										
N alam de										
3 hrs	D	3	1	0	0	0	0	0	0	
+						(I)	(I)	(I)	(I)	(I)
L-DOPA	G	0	2 1/2	2 1/2	2	2	3 in 2/4	3 in 1/4	2 in 1/4	G in a few a
40 mg kg	N	3	4	4	4	4	4	4	4	
NSD 1015	D	2 1/2	2 1/2	2	1/2	0	0	0	0	(II) in 4/6 G in
1 hr										a few a in 1/6
+				(II)	(III)	(I)	(I)			G in 10 % of a
L-DOPA 40	G	0	0	3	3	3	2	2 in 1/3	2 in 1/3	in 1/6 G in
mg kg										30 % of a
	N	6	6	6	6	7	3	3	3	
β thujaplicin	D				0	0				(III) in 5/7 G
1 hr										in a few a
+	G				2	2				in 2/7 G in
L-DOPA										60 % of a
40 mg kg	N				3	3				

acinar cells than in the surrounding connective tissue or in the acinar cells of non injected control animals shortly after L- or D DOPA injection This clearly suggests that there are active uptake mechanisms for L and D DOPA in the pancreatic acinar cells For the same reason it is probable that structure analogues such as L-α methyl DOPA DL-m tyrosine and DL-α methyl m tyrosine are also actively taken up and concentrated by the exocrine pancreatic cells It is of interest to note that the 3 hydroxylated derivatives of phenylalanine (m tyrosine and α methyl m tyrosine display a yellowish green colour in the exocrine pancreas whereas these substances give rise to a very short wave almost invisible blue fluorescence when localized to adrenergic nerves Jonsson and Ritzen 1966) From the present work and that of others (*Dahlstrom and Fuxe 1964 Sachs 1965 Hamberger 1967*) it seems that nerve cells and fibres are unique in this respect

In the studies on the pancreas there seems to be none devoted to the difference in the uptake of the D and L-stereoisomers of amino acids In other organs however such measurements have been made Generally both of the stereoisomers are considered to be actively taken up although the D isomers to a lesser extent than the L-isomers (Meister 1965) However the proportions vary between different organs and different amino acids From the present work it seems clear that the two stereoisomers of DOPA are actively taken up into the pancreas The proportions between the two stereoisomers can only be very roughly estimated but seem here to be approximately *equal*

The uptake of D and L-DOPA has recently been studied in a number of 'aminergic cell systems The enterochromaffin like cells of the rat gastric mucosa are able to take up both stereoisomers, and thus resemble the pancreas in this respect (Håkanson, Lilja and Owman 1967) On the other hand, only L-DOPA is being actively taken up and retained in significant amounts in a number of other systems, such as certain special cells in the mouse bronchial mucosa (Larson Owman and Sundler, to be published), the islets of Langerhans in rats and mice (Cegrell *et al* 1964, Cegrell 1968), the parafollicular cells of the mouse thyroid (Larson Owman and Sundler 1966) and the endothelial cells in the capillaries of the mouse brain (Bertler *et al* 1966)

Different metabolic pathways exist for amino acids taken up into the exocrine pancreatic cells A very prominent one is the incorporation into enzyme proteins followed by transport to the apical region of the cell and storage in the zymogen granules (see, *e g* Jamieson and Palade 1967) In mice and rats fed *ad libitum* with water and pellets, this procedure takes approximately 40—60 min (Nadler 1963, Warshavsky Leblond and Droz 1963, Van Heyningen 1964) Interestingly, this is also the time that elapses after the injection of L-DOPA for the appearance of the marked granular fluorescence However, this is somewhat remarkable since so far incorporation of L-DOPA into proteins has not been described nor is L DOPA known to be a significant constituent of proteins (*cf* Meister 1965b)

In view of the known occurrence of DOPA decarboxylase in the pancreas of different species (Holz, Credner and Strubing 1942) it was judged interesting to study the effect of decarboxylase inhibitors on the intracellular behaviour of DOPA It was then discovered that after such inhibition the appearance of the granular fluorescence was delayed The delay was prolonged by the repeated injection of NSD 1015 This indicates that the apical granules store DA rather than DOPA a suggestion strengthened by the observation that the injection of D DOPA (which is resistant to DOPA decarboxylase (see *e g* Lovenberg Weissbach and Udenfriend 1962)) did not result in any granular fluorescence Chemical analyses also strongly support this view (Alm and Rosengren to be published) Thus there seems to be a previously unsuspected mechanism in the zymogen granules namely to store a biogenic monoamine probably dopamine Upon administration of (H^3) 5 HTP 5 HT has likewise been shown to accumulate in the apical region of the exocrine mouse pancreatic cell (Gershon and Ross 1966) and is actually stored in apical granules (Alm to be published) Judged from the fluorescence of the granules the other structure analogues studied (L-α methyl DOPA DL m tyrosine and DL-α methyl m tyrosine) are not incorporated into the granules despite the fact that the L-isomers can be decarboxylated to the corresponding amines (see *e g* Lovenberg *et al* 1962 Carlsson and Lindqvist 1962 Porter and Titus 1963 Shore Busfield and Alpers 1964) This could suggest a strict chemical specificity of the uptake mechanism of the zymogen granules On the other hand the structure analogues are not decarboxylated as readily as L DOPA (Lovenberg *et al* 1962) and this could also explain why no fluorescent granules appear

The observations that the pancreatic zymogen granules probably take up and store DA raised the question whether the pancreatic acinar cells have a specific mechanism for taking up DA too. However, this is apparently not so. Even after huge i.v. doses of DA only little fluorescence appeared in the pancreas. The absence of fluorescence could possibly be the result of a very rapid break down of intracellular DA. However, the experiments did not suggest any very rapid disappearance of intracellular DA. *It thus seems that pancreatic acinar cells have no efficient mechanism for taking up DA.* In this respect, they resemble certain other cell systems that store monoamines, *such as pancreatic islets in mice and rats* (Cegrell *et al.* 1964, Cegrell 1968), monoamine storing cells in the mouse bronchial mucosa (Larson *et al.*, to be published), the enterochromaffin-like cells in the rat gastric mucosa (Håkanson *et al.* 1967) and endothelial cells of the mouse brain (Bertler *et al.* 1966).

It is generally believed that most of the proteins of the pancreatic juice stem from the zymogen granules, which are regarded as a cellular store for the proteins (*cf.* Palade, Siekevitz and Caro 1962). It would be tempting to assume that the DA is excreted directly with the granules. However, specific fluorescence has been observed in the pancreatic ducts only after excessive doses of L-DOPA (500 mg/kg and more). Moreover, the pancreas is known to contain both MAO (rat: West 1958, Petkov 1965) and COMT (monkey: Axelrod, Albers and Clemente 1959). Indeed the fact that inhibition of MAO and COMT cause the fluorescent zymogen granules to persist much longer than without enzyme inhibition seems to imply that these enzymes degrade the DA. If the degradation products are secreted into the pancreatic ducts, it readily explains the appearance of radioactivity in rat pancreatic juice after the injection of DL-DOPA C^{14} despite the fact that no specific fluorescence has been observed in the ducts (Alm, unpublished observations).

Some of the enzyme inhibitors used in this work have been described as inhibiting other steps in the metabolism of catecholamines than those indicated above. In rat epiphysis nialamide has a decarboxylase inhibitory effect (Håkansson and Owman 1965). However, in the rat pancreas treated with nialamide or 4-isopropyltropolone the granular fluorescence appears at the same time as after L-DOPA only. Thus, obviously, the decarboxylase inhibiting effect these substances have is not sufficient to interfere with the present experiments. Further, it is not known to what extent nialamide can affect the COMT activity or 4-isopropyltropolone the MAO activity, but any such interactions are without importance for the conclusions drawn.

This work was supported by grants from the Swedish Medical Research Council (B68-12X-712-03B and B68-14X-56-04B), the United States Public Health Service (06701-01), and the Faculty of Medicine, University of Lund, Lund, Sweden.

References

ALM, P., L. CEGRELL, B. EHINGER and B. FALCK, Remarkable adrenergic nerve fibres in the exocrine pancreas. *Z. Zellforsch.* 1967. *83.* 178—186.

ALM, P., B. EHINGER and B. FALCK, L-DOPA turnover in the mouse pancreas. *Life Sci.* 1967. *6.* 913—917.

AXELROD, J., W. ALBERS and C. D. CLEMENTE, Distribution of catechol-O-methyl transferase in the nervous system and other tissues. *J. Neurochem.* 1959. *5.* 68—72.

BERLINGUET L, N BEGIN and L M BABINEAU Autoradiographic studies of the distribution of 1 aminocyclopentane carboxylic acid in normal and cancerous mice *Canad J Biochem* 1962 *40* 111—1114

BERTLER, A, B FALCK and CH OWMAN Studies on 5 hydroxytryptamine stores in pineal gland of rat *Acta physiol scand* 1964 *63* Suppl 239

BERTLER A, B FALCK, CH OWMAN and E ROSENGREN, The localization of monoaminergic blood brain barrier mechanisms. *Pharmacol Rev* 1966 *18* 369—385

BLAU M and R F MANSKE, The pancreas specificity of Se^{75} selenomethionine *J nucl Med* 1961 *2* 102—105

BUSCH H, J R DAVIS G R HONIG D C ANDERSON P V NAIR and W L NYHAN The uptake of a variety of amino acids into nuclear proteins of tumours and other tissues *Cancer Res* 1959 *19* 1030—1039

CARLSSON A and M LINDQVIST, In vivo decarboxylation of α methyl DOPA and α methyl metatyrosine *Acta physiol scand* 1962 *54* 87—94

CASSANO G B and E HANSSON Uptake of (^{14}C) glutamine in the tissues of the mouse [illegible] *J Neurochem* 1965 *12* 851—855

[illegible] monoamines in the mammalian pancreas *Acta* [illegible]

[illegible] Monoaminergic mechanisms in the endocrine pancreas In *The structure and metabolism of the pancreatic islets* Ed S E Brolin B Hellman and H Knutson Pergamon Press 1964 p 429—435

CORRODI H and G JONSSON The formaldehyde fluorescence method for the histochemical demonstration of biogenic monoamines A review on the methodology *J Histochem Cytochem* 1967 *15* 65—78

DAHLSTROM A and K FUXE Evidence for the existence of monoamines containing neurons in the central nervous system I Demonstration of monamines in the cell bodies of brain stem neurons *Acta physiol scand* 1964 *62* Suppl 232

FALCK B Observations on the possibilities of the cellular localization of monoamines by a fluorescence method *Acta physiol scand* 1962 *56* Suppl 197

FA[illegible]

FA[illegible]

G[illegible] *Experientia* (Basel) 1962 *18* 43—44

GERSHON M D and L L ROSS Location of sites of 5-hydroxytryptamine storage and metabolism by radioautography *J Physiol* (Lond) 1966 *186* 477—492

HAMBERGER, B Reserpine-resistant uptake of catecholamines in isolated tissues of the rat. *Acta physiol scand* 1967 Suppl 295

HANSSON E The formation of pancreatic juice proteins studied with labelled amino acids *Acta physiol scand* 1959 *46* Suppl 161

HANSSON E and S T JACOBSSON Uptake of (^{75}Se) selenomethionine in the tissues of the mouse studied by whole body autoradiography *Biochim biophys Acta* (Amst) 1966 *115* 285—293

HOLZ P, K CREDNER and CH STRUBING Über das Vorkommen der Dopadecarboxylase im Pankreas *Naunyn-Schmiedeberg's Arch exp Path Pharmak* 1942 *199* 145—152

HAKANSSON R, B LILJA and CH OWMAN Properties of a new system of amine storing cells in the gastric mucosa of the rat *Europ J Pharmacol* 1967 *1* 188—199

HÅKANSSON R and CH OWMAN Effect of denervation and enzyme inhibition on DOPA decarboxylase and monoamine oxidase activities of rat pineal gland *J Neurochem* 1965 *12* 417—429

JAMIESON J D and G E PALADE Intracellular transport of secretory proteins in the pancreatic exocrine cell I Role of the peripheral elements of the Golgi complex. *J Cell Biol* 1967 *34* 577—596

JONSSON G and M RITZEN Microspectrofluorometric identification of metaraminol in sympathetic adrenergic neurons *Acta physiol scand* 1966 *67* 505—513

LARSON B, CH OWMAN and F SUNDLER Monoaminergic mechanisms in parafollicular cells of the mouse thyroid gland *Endocrinology* 1966 *78* 1109—1114

LOVENBERG W, H WEISSBACH and S UDENFRIEND Aromatic L amino acid decarboxylase *J biol Chem* 1962 *237* 89—93

MEISTER A Transport of amino acids In *Biochemistry of the amino acids* Ed A Meister Academic Press New York London 1965 a I 264—283

MEISTER A Other natural amino acids In *Biochemistry of the amino acids* Ed A Meister, Academic Press New York London 1965b I 101

NADLER N J Calculations of the turnover times of proteins in each region of the acinar cells of the pancreas *J Cell Biol* 1963 *16* 24—27
NORBERG K A Transmitter histochemistry of the sympathetic adrenergic nervous system *Brain Research* 1967 *5* 125—170
NORBERG K A M RITZEN and U UNGERSTEDT Histochemical studies on a special catechol amine containing cell type in sympathetic ganglia *Acta physiol scand* 1966 *67* 260—270
PALADE, G E PH SIEKEVITZ and L G CARO Structure chemistry and function of the pancreatic exocrine cell In *CIBA foundation symposium on the exocrine pancreas* J and A Churchill Ltd London 1962
PETKOV P De l activite monoaminoxydasique dans le pancreas de l homme et de certains mammifères rat blanc cobaye chat et lapin *Ann Histochim* 1965 *10* 17—24
PORTER C C and D C TITUS Distribution and metabolism of methyldopa in the rat *J Pharmacol exp Ther* 1963 *139* 77—87
RITZÉN M *Cytochemical identification and quantitation of biogenic monoamines* MD thesis Kungl boktryckeriet P A Norstedt & Soner Stockholm 1967
RITZEN M L HAMMARSTROM and S ULLBERG Autoradiographic distribution of 5 hydroxy tryptamine and 5 hydroxytryptophan in the mouse *Biochem Pharmacol* 1965 *14* 313—321
ROSELL S G SEDVALL and S ULLBERG Distribution and fate of dihydroxyphenylalanine-2 ^{14}C (DOPA) in mice *Biochem Pharmacol* 1963 *12* 265—269
SACHS CH Demonstration of metaraminol in nervous and non nervous tissues by a histochemical fluorescence reaction *Biochem Pharmacol* 1965 *14* 1773—1778
SHORE P A D BUSFIELD and H S ALPERS Binding and release of metaraminol Mechanism of norepinephrine depletion by a methyl m tyrosine and related agents *J Pharmacol exp Ther* 1964 *146* 194—199
VAN HEYNINGEN H E Secretion of protein by the acinar cells of the rat pancreas as studied by electron microscopic radioautography *Anat Rec* 1964 *148* 485—489
VIALLI M Histology of the enterochromaffin cell system In *Handbook of experimental pharmacology* Vol XIX 5 Hydroxytryptamine and related indolealkylamines Springer Verlag Berlin—Heidelberg—New York 1966
WARSHAWSKY H C P LEBLOND and B DROZ Synthesis and migration of proteins in the cells of the exocrine pancreas as revealed by specific activity determination from radioautographs *J Cell Biol* 1963 *16* 1—23
WEST G B 5 hydroxytryptamine and hyperglycemia *Nature* (Lond) 1958 *182* 182—183

Acta physiol. scand. 1969. 76. 121—130

From the Institute of Physiology, University of Oslo, Norway

Hypoxia and Pulmonary Vascular Resistance. The Relative Effects of Pulmonary Arterial and Alveolar P_{O_2}

By

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Received 7 August 1968

Abstract

HAUGE, A *Hypoxia and pulmonary vascular resistance The relative effects of pulmonary arterial and alveolar P_O*, Acta physiol scand 1969 *76 121—130*

The relative effects of pulmonary arterial and alveolar P_{O_2} on pulmonary vascular resistance (PVR) have been studied in isolated and ventilated rat lung preparations The experimental arrangement included two such preparations perfused in series with homologuous blood Pulmonary arterial hypoxemia (P_{O_2} 28—44 mm Hg) without alveolar hypoxia did not elicit vaso-

the main denominator for the vasoconstriction effect The results have been discussed in relation to the possible shape of the P_{O_2} gradient from alveolar gas to lumen of small resistance vessels

There is general agreement among investigators in the field that ventilation hypoxia elicits increased pulmonary vascular resistance in several mammalian species Direct evidence has been presented which indicates that constriction of arterial vessels is involved in the response (Kato and Staub 1966) Much less is known about the intrinsic mechanism triggered by the hypoxic stimulus The possibilities of hypoxia releasing or activating a local vasoactive substance as well as that of a more direct effect of reduced oxygen tension on the vascular smooth muscle have been suggested Bergofsky and Holtzman (1967) reported a reversible loss of potassium from pulmonary arterial strips under hypoxic conditions They calculated that this would correspond to a partial depolarization of the vascular smooth muscle cells bringing them closer to their excitatory threshold These authors suggest that this change, at least in part, is the mechanism responsible for the pulmonary vasoconstrictor response during hypoxia The findings of Hauge (1968a) and of Haavik Nilsen and Hauge (1968) seemed to indicate that interference with the metabolism of a vasoactive component in the lung, possibly histamine, could be involved

with constant volume pulsatile inflow (Hauge 1968 b) using a Harvard Medical Instruments dual peristaltic pump model 1210 The blood entered the preparations through cannulas inserted through the right ventricles into the pulmonary artery and drained into two thermostated blood reservoirs by way of cannulas inserted into the left ventricles The pulmonary arterial pressure [illegible] pressure trans [illegible], Burbank Cali [illegible] blood (Pa_{O_2}) of [illegible] ed and recorded continuously with a Beckman micro oxygen electrode connected through a Physiological Gas [illegible]

ature was kept at 37 C

Cross perfusion between the two pair of lungs was established by allowing the venous tubing of preparation 1 to drain into the blood reservoir of preparation 2 At the same time the venous tubing from preparation 2 was drained into the blood reservoir of preparation 1 The effluent blood from each lung preparation was pumped into the arterial tree of the other one

Ventilation The two lung preparations were ventilated independently by positive pressure inflation preparation 1 with a frequency of 20 per min For preparation 2 ventilation frequency was also in most cases kept at 20 per min In some experiments the frequency was changed and this could be carried out without interrupting the ventilation Peak inflation pressure was kept at 11 cm of water and expiration pressure at 2—4 cm of water for both preparations The ventilation overflow was recorded continuously on a kymograph as described by Konzett and Rossler (1940) Gentle hyperinflations were done at intervals of about 15 min and there were then no change in compliance over a period of several hours

T[illegible] ing test mixtures wer[illegible] 3) 96 % N_2 and 4 %[illegible] (the 21 % O_2 gas

Results

The two isolated and separately ventilated pair of lungs could be perfused either independently and in parallel, or in series (cross perfusion) It was thus possible to apply a low oxygen stimulus to a pair of lungs via the airways as well as via the blood The two routes of low oxygen stimulation were tested separately and in combination

In the first set of experiments one of the two pair of lungs (no 2) was ventilated with a hypoxic gas mixture (2 % O_2 or 0 % O_2) the other (no 1) with a normoxic gas mixture (21 % O_2) Fig 1 demonstrates the effects of changing from independent perfusion to cross perfusion for these two preparations During cross perfusion lungpair 2 received arterial blood of a high P_{O_2} but hypoxic ventilation gas, whereas lung pair 1 received hypoxic arterial blood but a normoxic ventilation gas The pulmonary vascular resistance (PVR) of lung pair 1 remained unchanged and low, as indicated by the unaltered pulmonary artery pressure (P_{PA}) In lung pair 2 a rise in P_{PA} had been going on as a result of the previously started ventilation hypoxia After starting cross perfusion this rise stopped and the pressure slowly began to return towards control level When independent parallell perfusion of the two pair of lungs were reestablished the P_{PA} of lung pair 2 resumed its upward trend until its ventilation gas was changed back to the 21 % O_2 mixture

In 5 lung pairs receiving blood from the deoxygenating lungs the pulmonary arterial oxygen tension (Pa_{O_2}) stabilized at levels which varied between 28 mm

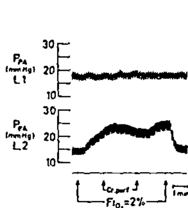


Fig 1

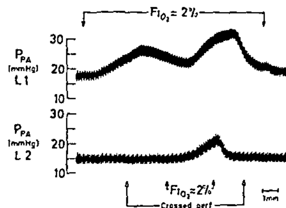


Fig 2

Fig 1 Effects of cross perfusion (Cr perf) of two isolated rat lung preparations while one (L 1) was ventilated with 21 % O_2 the other (L 2) with 2 % O_2 as indicated by arrows Control ventilation gas 21 % O_2 P_{PA} pulmonary arterial pressure F_{IO_2} fraction of oxygen in dry ventilation gas

Fig 2 Two isolated perfused rat lung preparations One (L 1) was ventilated with 2 % O_2 through out a period of cross perfusion the other (L 2) for a part of this period, as indicated by arrows Control ventilation gas 21 % O_2 Symbols and abbreviations as in Fig 1

Hg and 44 mm Hg As long as these lungs were ventilated with the 21 % O_2 gas mixture, this level of prealveolar hypoxemia did not cause significant changes in PVR The same lungs reacted, however with brisk pressor responses to ventilation hypoxia Such pressor responses to ventilation hypoxia were fully reversible also when the Pa_{O_2} was continuously kept low (Fig 2)

Pulmonary arterial hypoxemia alone, without airway hypoxia, could thus apparently not elicit a vasopressor response, whereas increases in Pa_{O_2} interfered with the pressor response to simultaneous alveolar hypoxia The oxygen tension of the perfusate blood from lungs ventilated with the 21 % O_2 gas mixture was usually about 120 mm Hg The introduction of such blood into the prealveolar vascular tree reduced the pressor response to ventilation hypoxia in all experiments of this type Fig 1 and 2 This effect was not, or only slightly, more pronounced *when the donor lungs producing the hyperoxic blood were ventilated with a 96 %* O_2 gas mixture thereby elevating the Pa_{O_2} of the recipient lungs to above 300 mm Hg At such very high levels of Pa_{O_2} ventilation hypoxia in the recipient pair of lungs still maintained a PVR well above control level (Fig 3

Fig 4 demonstrates the resultant pressor effects of ventilating the same pair of lungs for successive 2 min periods with the 2 % O_2 gas mixture whilst the Pa_{O_2} was kept at levels of 112 350 and 38 mm Hg respectively A continuous cross circulation with another lung pair was established and the Pa_{O_2} of the test lungs was set at the three different levels by appropriate selection of the gas mixture for the other, blood-equilibrating lung pair The pressor response to ventilation hypoxia was again considerably reduced at the high levels of Pa_{O_2} This reduc

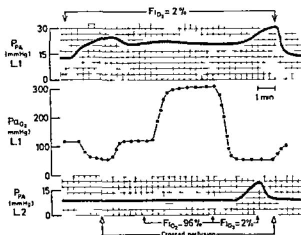


Fig 3 Two isolated perfused rat lung preparations One (L 1) was ventilated with 2 % O_2 throughout a period of cross-perfusion, the other (L 2) with 21 % O_2 (control gas), 96 % O_2, 2 % O_2 and 21 % O_2 in sequence, as indicated Also shown is pulmonary arterial oxygen tension (Pa_{O_2}) of L 1 Symbols and abbreviations as in Fig 1

tion was, however, not significantly greater at the Pa_{O_2} level of 350 than at that of 112 mm Hg One explanation of this finding could be that it is primarily the oxygen content of the blood perfusing the pulmonary arterial tree that influences the magnitude of the pressor response to ventilation hypoxia During ventilation hypoxia perfusate blood of high oxygen content will give off oxygen to the alveoli, and thereby interferring with the alveolar oxygen tension as a vasoconstrictor stimulus When blood flow and ventilation are kept constant, the magnitude of this interference will primarily depend on the oxygen content of the perfusate blood

To test this hypothesis the effect of changing the level of alveolar ventilation was tested, whilst keeping the high Pa_{O_2} and the composition of the hypoxic gas mixture constant An experiment of this this type is demonstrated in Fig 5 Lung-pair 1 was ventilated with the 96 % O_2 gas mixture, lung pair 2 with the 2 % O_2 gas mixture When a pressor response was apparent in lung-pair 2, cross-perfusion was established This brought the Pa_{O_2} of lung-pair 2 to 310 mm Hg and caused a reduction in the elevated PVR of this lung-pair During the next 6 min ventilation frequency (f) of lung-pair 2 was twice temporarily increased from 20 to 40 per min Such an increase in ventilation caused a rapid rise in PVR Return to low frequency ventilation each time reversed the effect Similar results were obtained in two other experiments of the same type In one of these, effluent or venous blood

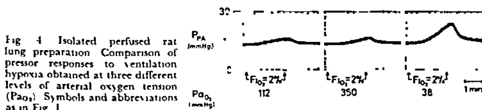


Fig 4 Isolated perfused rat lung preparation Comparison of pressor responses to ventilation hypoxia obtained at three different levels of arterial oxygen tension (Pa_{O_2}) Symbols and abbreviations as in Fig 1

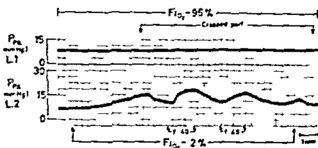


Fig 5 Two isolated perfused rat lung preparations One (L 1) was ventilated with 95 % O_2, the other (L 2) with 2 % O_2 as indicated. During a period of crossed perfusion, ventilation frequency (f) of L 2 was twice elevated from the standard 20/min to 40/min causing reversible increases of pulmonary arterial pressure Pulmonary arterial oxygen tension of L 2 was 320 mm Hg Abbreviations and symbols as in Fig 1

oxygen tension (P_{VO_2}) of lung pair 2 was recorded The increase in ventilation frequency caused a reversible fall in P_{VO_2} of 4 mm Hg Similar changes in ventilation frequency during normoxic ventilation had no effects on PVR or on P_{VO_2}

Fig 6 demonstrates the effect of a stepwise change in ventilation frequency during ventilation with the 2 % O_2 gas mixture The Pa_{O_2} was maintained at 120 mm Hg throughout, by another pair of lungs At the initial ventilation frequency of 20 per min the preparation showed a moderate increase in PVR as indicated by the slowly rising P_{PA} The rate of rise in P_{PA} increased progressively with the two stepwise changes in ventilation frequency to 40 and 54 The rise in PVR stopped immediately upon return to the initial ventilation frequency of 20 per min, and PVR then started to fall slowly The experiments of Fig 5 and 6 demonstrate that an effect of high Pa_{O_2} on the pressor response to ventilation hypoxia can be counteracted by increases in alveolar ventilation This is in agreement with the hypothesis of arterial blood effects being mainly related to changes in alveolar P_{O_2}

Another set of experiments were carried out in order to examine this relationship Here lung pairs exposed to moderate pulmonary arterial hypoxemia were followed during periods of no renewal of the alveolar gas An experiment of this type is depicted in Fig 7 One lung pair (tracing not shown) was ventilated with the 2 % O_2 gas mixture the other lung pair with the 21 % O_2 gas mixture and cross-perfusion established Pa_{O_2} of the hypoxic blood recipient was about 35 mm Hg As usual this caused no pressor response When the tracheal tubing of this lung pair was clamped however a pressor response appeared after a delay period of

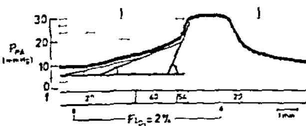


Fig 6 Isolated perfused rat lung preparation Effects of ventilation hypoxia at three different levels of ventilation frequency (f) maintaining a constant pulmonary arterial blood oxygen tension of 120 mm Hg Abbreviations and symbols as in Fig 1

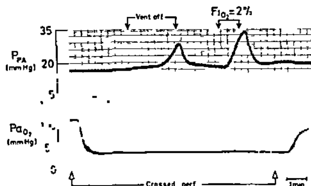


Fig 7 Isolated perfused rat lung preparation ventilated with 21% O_2 The preparation received hypoxic blood from another pair of lungs (not shown) ventilated with 2% O_2, indicated as Crossed perf No pressor response was elicited in the hypoxic blood recipient until ventilation was temporarily stopped (Vent. off) Also shown is a pressor response to ventilation hypoxia (2% O_2) superimposed on arterial hypoxemia and pulmonary arterial oxygen tension (Pa_{O_2})

about 1 min On restarting the ventilation P_{PA} returned to its control level Also demonstrated is the pressor effect of a subsequent short period of ventilation hypoxia The same type of effects could be repeatedly obtained in all of 4 pair of lungs in which such tests were carried out In 1 expt with tracheal clamping the oxygen electrode was placed on the venous side of the preparation After clamping of the tracheal tubing in inspiratory phase it took 5 min until equilibrium between alveolar P_{O_2} and Pv_{O_2} was obtained, as indicated by stable Pv_{O_2} When trachea was clamped in the expiratory phase it took about 3 1/2 min At the time when the pressor response could be observed, Pv_{O_2} was about 65 mm Hg Clamping of trachea at high Pa_{O_2} during normoxic ventilation gave no change in PVR

Whether a rise in Pa_{O_2} will induce a rise in Pv_{O_2} during ventilation with the 2% O_2 depends on the capacity of the lungs as gas equilibrators This again will mainly depend upon the magnitude of ventilation relative to that of blood flow In 1 of 2 expts where oxygen measurements in effluent blood was carried out, Pv_{O_2} did not change upon alteration of Pa_{O_2} from 40 to 120 and then to 350 mm Hg as long as the lungs were ventilated with the 2% O_2 gas mixture In the other of these experiments Pv_{O_2} of lung pair 2 rose 4 mm upon the first of these changes in Pa_{O_2} No further change was seen when the highest Pa_{O_2} was introduced (f=20)

One recording only was made of Pv_{O_2} of a lung preparation ventilated with 96% O_2 An electrode reading of 640 mm Hg was observed, indicating very little shunting of blood

Discussion

The present experiments demonstrate that the oxygen tension of the pulmonary arterial blood is not without influence on PVR There was, however, a striking difference between the marked way in which low alveolar oxygen tension affects PVR and the moderate influence of Pa_{O_2} Admittedly the lung preparations in this series of experiments have been somewhat hyperventilated, and this might have exaggerated the domination of airway hypoxia over blood hypoxemia as a pressor stimulus

In the present preparation of an isolated rat lung a P_{IO_2} of 53 mm Hg (F_{IO_2} = 7 %) a potent pressor stimulus (Hauge 1968 b), and in the intact cat a reduction of $_{O_2}$ to 84 mm Hg (F_{IO_2} = 12 %) will elicit a rise in PVR (Hauge and Staub 69 A reduction of Pa_{O_2} in the isolated rat lung to somewhat less than 30 mm g did, however, not affect PVR as long as the lungs were ventilated with normoxic s

Teleologically it was hardly surprising to find Pa_{O_2} relatively ineffective in elic ng PVR increases It would be most disadvantageous if low pulmonary Pa_O ring for instance, heavy exercise, should cause an additional work load on the ht ventricle Pulmonary arterial hypoxemia is, however, able to potentiate the essor effect of airway hypoxia Such an interaction may be of importance for the development of the very high pulmonary artery pressures which has been corded in man after strenuous exercise at high altitudes (Banchero *et al* 1966) ixed venous P_{O_2} during exercise at 3450 m has been reported to be as low as m 24 to 31 mm Hg Haab *et al* 1967)

The next question is how and where arterial oxygen tension and alveolar oxygen ision interact in determining PVR Boake *et al* (1959) found in the dog that usion of well oxygenated blood into the right auricle reduced the pressor response ventilation hypoxia They suggested that both the pulmonary vessels and the passages are sensitive to anoxia The present experiments have confirmed the ding that high Pa_O can reduce the pressor response to ventilation hypoxia. o sets of observation suggest, however, that this effect is accomplished mainly rough changes of P_{O_2} at some common extravascular site 1 Efforts to maintain hypoxic alveolar gas composition by hyperventilation counteracted the damp- g effect of a concomitantly high Pa_{O_2} 2 The PVR reducing effect of high $_{O_2}$ was about the same whether Pa_{O_2} was 120 or 320 mm Hg This latter finding ggests that the amount of oxygen which can be given off during the passage of ood through the hypoxic lungs determines the magnitude of this effect It makes less likely that a direct action of high Pa_O on the vascular smooth muscle is olved

In this series of experiments pulmonary arterial oxygen tension did not rise much ove 300 mm Hg in lungs receiving blood from a preparation ventilated with the % O_2 gas mixture Since Pa_{O_2} stabilized under these conditions it is unlikely at the effect is caused solely or predominantly by shunting of blood through e hyperoxic lung The relatively low Pa_{O_2} is presumably for the main part due oxygen loss in the venous tubings and in the blood reservoir With a blood flow 10 ml min and a blood volume from 10 to 18 ml there was ample time for exposure room air Such oxygen loss was not felt to be a disadvantage as long as the pul onary arterial oxygen tension of the recipient lung was known and recorded

Several observations indicate that it is oxygen tension in or near the alveoli hich determines the degree of the pressor response When normoxic ventilation as stopped 1 1/2 to 2 min elapsed before arterial hypoxemia elicited a pressor sponse At this point alveolar hypoxia was clearly present demonstrated by falling

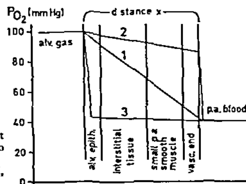


Fig 8 A model demonstrating three different oxygen tension gradients from alveolar gas to lumen of small resistance vessel alv epith alveolar epithelium p a pulmonary arterial, vasc. end vascular endothelium

P_{vO_2} It is also most likely that the effector area is localized vascularly upstreams to the alveoli, since changes in Pa_{O_2} which either potentiated or reduced the press response to ventilation hypoxia did so in spite of constant or almost constant P_{vO_2}

The present experiments alone cannot tell whether the "receptor" is localized near gas or blood To answer this question one has to know the shape of the P_{O_2} gradient from the alveolar wall to vessel lumen of small pulmonary arteries, and also how this gradient is affected by the experimental procedures Fig 8 demonstrates three different P_{O_2} gradients for intact animal, drawn on a model of tissue cross section, 1) a linear fall, 2) a flat curve steep at blood interface, and 3) a steep curve at air interface, then flat towards blood

If curve 1 gives a correct picture of the P_{O_2} gradient, then receptor site must be closer to alveolar than to vessel lumen to be preferentially affected by alveolar hypoxia But this curve is unlikely to be correct as shown by data on gas penetration in pulmonary vessels (Sobol *et al* 1963, Jameson 1964) Oxygenized blood is found in pulmonary arteries of considerable size (Staub 1961) If curve 3 is correct, then regardless of receptor site, unless it is on alveolar surface, blood O_2 tension must preferentially affect the response This is contradicted by evidence in this paper and also by the gas penetration data mentioned above If curve 2 is correct, then alveolar gas P_{O_2} will be the main determinant of the response regardless of receptor location, unless the receptor should be floating in the pulmonary arterial blood With a normal P_{O_2} gradient of this shape the present data cannot give information of the nearness of the receptor to blood or gas This curve is the only one which is compatible both with the earlier observations on gas penetration and the present results The distance x on the figure is only about 10 to 20 μ for pulmonary arteries of internal diameter of 100 μ (Staub personal communication)

References

BANCHERO N F SIME D PENALOZA J CRUZ R GAMBOA and E MARTICORENA Pulmonary pressure cardiac output and arterial oxygen saturation during exercise at high altitude and at sea level *Circulation* 1966 33 249—262

BERGOFSKY E H B G BASS R FERRETTI and A P FISHMAN Pulmonary vasoconstriction in response to precapillary hypoxemia *J clin Invest* 1963 42 1201—1215

BERGOFSKY, E H and S HOLTZMAN, A study of the mechanism involved in the pulmonary arterial pressor response to hypoxia *Circulat Res* 1967 *20* 506—519
BOAKE W C, R DALEY and I K R MCMILLAN, Observations on hypoxic pulmonary hypertension *Brit Heart J* 1959 *21* 31—39
CASSIN S, G S DAWES and B B ROSS, Pulmonary blood flow and vascular resistance in immature foetal lambs *J Physiol* (Lond) 1964 *171* 80—89
DUKE, H N, The site of action of anoxia on the pulmonary blood vessels of the cat *J Physiol* (Lond) 1954 *125* 373—382
HAAB, P, A CHINET and L E FARHI Changes in mixed venous oxygen tension at rest and exercise during exposure to altitude In *Exercise at Altitude Based on papers presented at The international symposium, Milan, 1966*, ed by R Margaria Excerpta Medica Foundation, 1967 p 145—146
HAUGE, A Role of histamine in hypoxic pulmonary hypertension in the rat I Blockade or potentiation of endogenous amines, kinins and ATP *Circulat Res* 1968 a *22* 371—383
HAUGE, A, Conditions governing the pressor response to ventilation hypoxia in isolated perfused rat lungs *Acta physiol scand* 1968 b *72* 33—44
HAUGE, A and N C STAUB Prevention of hypoxic vasoconstriction in cat lung by histamine releasing agent 48/80 Submitted to *J appl Physiol* 1969
HAAVIK NILSEN, K and A HAUGE, Effects of temperature changes on the pressor response to acute alveolar hypoxia in isolated rat lungs *Acta physiol scand* 1968 *73* 111—120
JAMESON, A G, Gaseous diffusion from alveoli into pulmonary arteries *J appl Physiol* 1964 *19* 448—456
KATO M and N C STAUB Response of small pulmonary arteries to unilobar hypoxia and hypercapnia *Circulat Res* 1966 *19* 426—440
KONZETT H and R ROSSLER Versuchsanordnung zu Untersuchungen an der Bronchialmuskulatur *Naunyn-Schmiedebergs Arch exp Pat Pharmak* 1940 *195* 71—74
NISELL, O The influence of blood gases on the pulmonary vessels of the cat *Acta physiol scand* 1951 *23* 85—90
REEVES J T J E LEATHERS B EISEMAN and F C SPENCER Alveolar hypoxia versus hypoxemia in the development of pulmonary hypertension *Med thorac* 1962 *19* 561—572
SOBOL, B J G BOTTEX C EMIRGIL and H GISSEN Gaseous diffusion from alveoli to pulmonar vessels of considerable size *Circulat Res* 1963 *13* 71—79
STAUB N C, Gas exchange vessels in the cat lung *Fed Proc* 1961 *20* 107

Acta physiol scand 1969. 76 131—136

From the Institute of Neurophysiology, University of Copenhagen Denmark

Response of Isolated Muscle Spindles of the Frog to Sinusoidal Changes in Length

By

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Received 8 August 1968

Abstract

Jahn, S Al Azharia *Response of isolated muscle spindles of the frog to sinusoidal changes in length* Acta physiol scand 1969 76 131—136

The response of isolated muscle spindles of the frog to sinusoidal vibrations was studied by subjecting the preparation to deformations of 0 15—1 mm at frequencies of 40—200 Hz. The [illegible] ation [illegible] o be [illegible] gion [illegible] le of [illegible] was [illegible] (T) [illegible]

The response of muscle spindles to vibratory stimuli has previously been studied by applying the stretch to the tendon of whole muscle or to the skin above the tendon (Echlin and Fessard 1938 Granit and Henatsch 1956 Bessou and Laporte 1962, Bianconi and van der Meulen 1963 Hagbarth and Eklund 1966 Rack 1966 Brown, Engberg and Matthews 1967) To obtain information about the threshold of the muscle spindle with the least possible interference from the visco-elastic properties of muscle tissue in parallel and in series with the receptor the experiments reported in this study were performed on isolated muscle spindles of the frog The minimum deformation to elicit an afferent response to each cycle was then determined for vibratory stimuli at different frequencies The deformation was expressed in terms of the displacement of the equatorial region and the threshold was compared with that obtained in experiments with transient stretches (Jahn 1968b and c)

Methods

Preparation The experiments were performed on isolated muscle spindles of the m extensor longus digiti IV of *Rana temporaria* at 17—20° C The dissection and the composition of the Ringer's solution have been described Jahn 1968a and b The "resting" muscle spindle was stretched 10—15 and 20—25 per cent above equilibrium length i.e the length at which the preparation is just taut

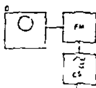


Fig 1. Schematic diagram of the stretch device for vibratory stimuli
AU laboratory generator
D differentiator (to improve frequency response)
A d c amplifier
BPM Brush pen motor
at aluminium tube
m vibratory movement
C condenser
OSC oscillator
FM FM detector
O oscilloscope
ch experimental chamber
t proximal tendon of the dissected extensor muscle
sp isolated muscle spindle

Stimulation and recording (Fig 1) Sinusoidal current at 10—260 Hz (Radiometer laboratory generator H 09 b) was transformed to alternating changes in length by feeding it into an electromagnetic device otherwise used as an ink writer (Brush pen motor BL 902) To improve the frequency response the writing pen was replaced by an aluminium tube 4 cm in length. The frequency response was determined by applying alternating current of 10—260 Hz of constant strength to the pen motor with the preparation connected to it The deformation was measured by a condenser myograph (Buchthal 1942) The movable plate of this myograph was attached to the proximal tendon of the dissected extensor muscle The changes in capacity were measured by a high frequency arrangement and displayed on one beam and alternating current on the other beam of an oscilloscope Within a frequency range of 160 Hz the amplitude of the vibrations was nearly constant (Fig 3 A) Below 40 and above 200 Hz the amplitude decreased. Between 160 and 200 Hz the vibration varied considerably in size The vibrator used in the present study did not allow to determine the maximum frequency of vibration at which muscle spindles are able to respond

The vibrational stimulus of a given frequency and amplitude was maintained for 200—300 msec and repeated 5—6 times at a rate of less than once every other minute The afferent action potentials were led off extra-cellularly with glass capillary microelectrodes filled with a 5 M NaCl solution The electrode was inserted through the capsule of the muscle spindle in the equatorial region as described (Jahn 1968a) The action potentials were amplified and displayed for recording on one beam of an oscilloscope simultaneously with the vibrations displayed on the second beam The traces were photographed from single sweeps 140—170 msec in duration

The condenser myograph was calibrated by slow movements of known amplitude applied to the aluminium tube of the myograph with the muscle spindle attached to it The change in capacitance was proportional to the displacement of the aluminium tube within a movement of 2 mm

The displacement of the equatorial region of the muscle spindle was measured by placing a graphite granule 10—20 μ in diameter on the spindle capsule At an initial stretch of 10—15 per cent a movement of the aluminium tube of 2 mm caused a displacement of the equatorial region of 380—400 μ at an initial stretch of 20 25 per cent the displacement was 180—200 μ The displacement of the graphite granule at different frequencies of vibration was measured by means of a stroboscope When the flashes were timed to occur at the top and the bottom of the vibration the two shadows of the graphite granule were farthest apart and their distance was measured by an ocular micrometer magnification × 400 and on microphotographs (Fig 2 The size of the granule determines the minimum displacement which can be measured 10—20 μ The measuring accuracy was increased to about 10 μ by using a well defined irregularity on the granule as mark of reference The measurements were performed on four spindles stretched 10—15 per cent and on two stretched 20 25 per cent The displacement was greatest at low frequencies and reached a minimum at 150 Hz Fig 3 B At this high frequency the deformation could no longer be discriminated in three muscle spindles

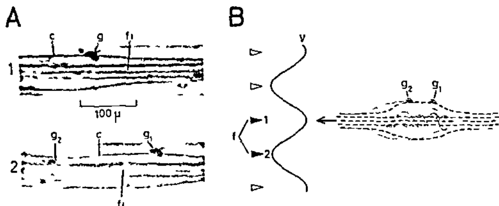


Fig 2 Stroboscopical measurement of the displacement of the equatorial region of the muscle spindle during sinusoidal vibrations

A 1) Graphite granule (g) on the surface of the spindle capsule (c)

2) "Shadows" of the graphite granule (g1, g2) during phasic stretches at 40 Hz with peak to peak amplitude of 1 3 mm measured at the aluminium tube of the stretch device

f1 intrafusal muscle fibres (focus on the surface of the muscle spindle)

B The greatest distance between the "shadows" g1 and g2 indicating the maximum displacement of the granule g, was obtained when the flashes (f) of the stroboscope were timed to occur at the top (2) and bottom (1) of the vibration (v)

To correct for small differences in the size of vibration applied to the different spindles, the displacement of the graphite granule was expressed in μ per mm movement of the condenser myograph with due consideration to the frequency response of the set up (Fig 3 A)

Results

Threshold as a function of the frequency of vibration Measured by the displacement of the aluminium tube of the stretch device (p 2) the minimum amplitude of vibration which elicited an afferent action potential in each cycle increased with frequency and reached maximum at 150—180 Hz (5 spindles stretched 10—15 per cent and 4 stretched 20—25 per cent) At 100 Hz the threshold averaged 270 μ when the initial elongation was 10—15 per cent and 600 μ when the initial elongation was 20—25 per cent (Fig 3 C) Measured by the displacement of the equatorial region the lowest threshold of deformation was obtained at 120—160 Hz and was 7—10 μ in spindles stretched 10—15 per cent, the same threshold as found when single transient stretches were applied (Jahn 1968b) With frequencies of 40—60 Hz the threshold was about twice the threshold for single transient stretch (Fig 3 D) When the initial length was 20—25 per cent above equilibrium length the displacement required to obtain a response to each cycle was greater than that for a single transient stretch 1 5 times at 120—160 Hz 3 times at 60—80 Hz and 2—3 times above 160 Hz (Fig 3 D)

When the amplitude of vibrations was below threshold the number of afferent responses decreased (Fig 4) With 70—80 per cent of the amplitude which elicited an afferent action potential in each cycle every second or third cycle evoked an action potential When the amplitude was less than 60 per cent the evoked action potentials could not be distinguished from spontaneous ones (Fig 5)

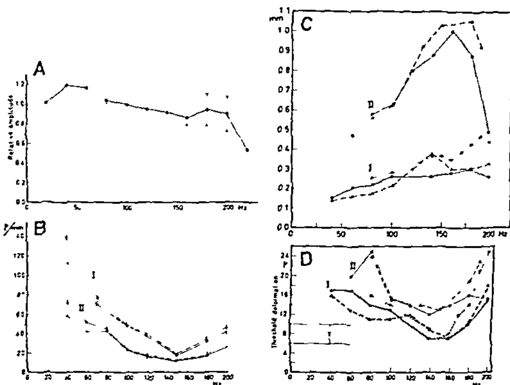


Fig 3 A Frequency response of the stretch device with the muscle spindle attached to it 4 different spindles [illegible]

B Average displacer[illegible] [illegible]ile of the muscle spindle as a function c[illegible] [illegible]ngation 10—15 per cent II 2 spindles [illegible] (full lines) and with correction for the variation of amplitude with frequency (cf A) (broken lines) Ordinate Displacement of the granule in μ per mm stretch of the muscle spindle

C and D The threshold amplitude of vibration which evoked a response as a function of frequency C deformation measured at the site of the aluminium tube of the stretch device, D deformation measured as the displacement of the equatorial region of the muscle spindles

I three spindles with an initial elongation of 10—15 per cent

II two spindles with an initial elongation of 20—25 per cent

"T" is the threshold deformation for single stretches

Discussion

The study of the response of amphibian muscle spindles to vibratory stimuli has demonstrated the role played by the visco-elastic properties of the tissue in series with the sensory ending The deformation of the total preparation required to elicit an afferent action potential in each cycle increased with the frequency of vibration the threshold at 150 Hz being about twice that at 40 Hz This is similar to findings in de-efferented mammalian muscle spindles *in situ* (Granit and Henatsch 1956) and to findings with trains of transient stretches (Jahn 1968*c*) However, measurement of the displacement of the equatorial region of the muscle spindle revealed that the increase in threshold with frequency was due to a decrease of the displacement of the equatorial region the displacement at 150 Hz being one fifth of that

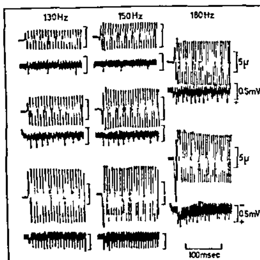


Fig 4 Increasing responsiveness of the muscle spindle to sinusoidal vibration (130, 150 and 180 Hz) when the amplitude was increased until every cycle just evoked an afferent action potential Upper trace displacement of the equatorial region of the spindle Lower trace afferent action potentials

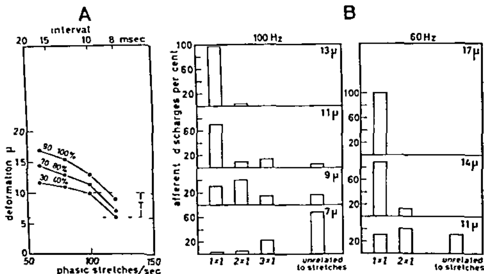


Fig 5 A Decrease of responsiveness with decreasing frequency of vibration The figures indicate the percentage of sinusoidal stretches which evoked an afferent action potential

Ordinate displacement of the equatorial region necessary to evoke the percentage of responses which is indicated above each curve (2 spindles, initial elongation 10—15 per cent 'T' is the threshold deformation for single transient stretches

B Distribution of the intervals between the action potentials in units of the period at 60 and 100 Hz for different deformations (7—17 µ 2 muscle spindles at an initial elongation of 10—15 per cent)

at 40 Hz In this comparison the frequency response of the stretch device was of minor importance since correction for it diminished the displacement of the equatorial region by 22 per cent at 40 Hz and increased it by 5—14 per cent at 120—160 Hz The displacement of the equatorial region obtained with slow stretches and

with vibrations of 40 Hz indicated that 75—90 per cent of the deformation took place in the elastic connections between the muscle spindle and the myograph. With single slow stretches the displacement of the equatorial region was 10—20 per cent of the externally applied deformation, with vibrations of 40 Hz it was 6—12 per cent and with 150 Hz it was 1—2 per cent. Thus the stiffness of the muscle spindle increased 5—8 times when the frequency of vibration was augmented from 40 to 150 Hz. In the same frequency range the stiffness of extrafusal muscle fibres increased three times (Buchthal, Kaiser and Knappeis 1944).

A decrease in displacement of the equatorial region can probably also explain the increase in threshold with increasing frequency found with trains of transient stretches (Jahn 1968c). When comparing the response to vibratory stimuli and to trains of transient stretches it must be born in mind that in the vibration experiments a change in deformation is always associated with a change in the velocity of stretch. These two parameters can only be varied independently if the frequency of vibration is changed as well.

In isolated Pacinian corpuscles threshold and frequency of vibration were similarly related as in the muscle spindle (deformation measured in the equatorial region). The minimum threshold occurred at 200 Hz and was the same as that for single stimuli with rectangular pulses (Sato 1961).

I wish to express my sincere gratitude to Professor F. Buchthal for constant advice and encouragement, to Poul Rosenfalck M. Sc. for valuable theoretical discussions and to Mr. V. Andersen for the design of the experimental arrangement.

The work was supported by the Muscular Dystrophy Associations of America Inc., New York and by the Danish State Research Foundation.

References

BESSOT, P. and Y. LAPORTE. Responses from primary and secondary endings of the same neuromuscular spindle of the tenuissimus muscle of the cat. *Symp. Muscle Receptors* pp. 105—119. ed. Parker D. Hong Kong Univ. Press 1962.

BIANCONI, R. and J. P. VAN DER MEULEN. The response to vibration of the end organs of mammalian muscle spindles. *J. Neurophysiol.* 1963. *26.* 177—190.

BROWN, M. C., I. ENGBERG and P. B. C. MATTHEWS. The relative sensitivity to vibration of muscle receptors of the cat. *J. Physiol.* (Lond.) 1967. *192.* 773—800.

BUCHTHAL, F. The mechanical properties of the single striated muscle fibre at rest and during contraction and their structural interpretation. *Dan. biol. Medd.* 1942. *17.* 140 pp.

ECHLIN, F. and A. FESSARD. Synchronized impulse discharges from receptors in the deep tissues in response to a vibrating stimulus. *J. Physiol.* (Lond.) 1938. *93.* 312—334.

GRANIT, R. and H. D. HENATSCH. Gamma control of dynamic properties of muscle spindles. *J. Neurophysiol.* 1956. *19.* 356—366.

HAGBARTH, K. E. and G. EKLUND. Motor effects of vibratory muscle stimuli in man. *Muscular Afferents and Motor Control.* pp. 177—186. ed. Granit, R. Almqvist and Wiksell. Stockholm 1966.

JAHN, S. AL AZHARIA. Spontaneous afferent discharges and spontaneous intrafusal contractions in isolated muscle spindles of the frog. *Acta physiol. scand.* 1968a. *72.* 350—365.

JAHN, S. AL AZHARIA. Response of isolated muscle spindles to single transient stretches. *Acta physiol. scand.* 1968b. *72.* 441—455.

JAHN, S. AL AZHARIA. The excitability of the frog's isolated muscle spindles during repetitive stretches. *Acta physiol. scand.* 1968c. *73.* 418—426.

RACK, P. M. H. The behaviour of a mammalian muscle during sinusoidal stretching. *J. Physiol.* (Lond.) 1966. *183.* 1—14.

SATO, M., Response of Pacinian corpuscles to sinusoidal vibration. *J. Physiol.* (Lond.) 1961. *159.* 391—409.

Acta physiol. scand. 1969. 76. 137—147

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The Role of Adenine Nucleotides in the Peripheral Vasodilating Effect of Thrombin

By

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Received 8 August 1968

Abstract

OLSSON, P., J SWEDENBORG and A C TEGER-NILSSON *The role of adenine nucleotides in the peripheral vasodilating effect of thrombin* Acta physiol scand 1969 *76* 137—147

The experiments were designed to study the mediating mechanism for the previously demon-

adenosine and adenine nucleotides caused the same type of reactions as that of vasoactive supernatants. By inactivation experiments where these substances as well as vasoactive supernatants were heated or incubated with plasma or ADP ase—ATP ase (Apyrase), it was concluded that the vasodilating agents in the latter are mainly ADP and to some extent ATP

In order to clarify the pathophysiology in states of disseminated intravascular coagulation we earlier studied the effects on the circulation caused by small amounts of thrombin Infusion of thrombin in amounts too small to cause intravascular clot formation into the femoral artery of a dog induced a marked local decrease in peripheral vascular resistance (Delin, Olsson and Teger-Nilsson 1967) A generalized decrease in the peripheral resistance also occurred when thrombin was infused intravenously into defibrinogenated animals These circulatory effects of thrombin were independent of products of the thrombin fibrinogen reaction, and did not seem to derive from any precursor in plasma Suspensions of unwashed blood cells in saline however became highly vasoactive after incubation *in vitro* with small amounts of thrombin (Olsson Swedenborg and Teger-Nilsson 1968)

The present work was designed to study the release of vasoactive material from washed blood cells suspended in saline and treated with small amounts of thrombin. After thrombin treatment and subsequent centrifugation, the vasoactive principle was found in the supernatant The effects of the supernatant were compared to those

of established vasoactive substances which might be released by thrombin from formed elements in blood Substances taken into consideration in this comparison were serotonin histamine, adenosine and adenine nucleotides

Reagents

Thrombin Commercial bovine thrombin from Parke Davis Co, Detroit Michigan U.S.A. The thrombin activity given by the firm was taken for granted

Adenosine Sigma Chemical Company, St Louis Missouri U S A

Adenin ... osphate (ADP)

Adenosine ... Missouri U S A

Seroton ... ians

Histam

Hepari

Before

Apyrase

Methods

Preparation of Vasoactive Material

Autologous homologous and heterologous (human) blood were used Autologous blood (150—200 ml) was drawn from the carotid artery immediately before the experiment Homologous and human blood were sampled by venous puncture the day before the experiments The withdrawal was made into polyethylene vessels containing one volume 0 13 M trisodium citrate for 9 volumes of blood All handling of blood during the preparations was made in polyethylene vessels The blood was centrifuged at 1000 g for 30 min in room temperature The plasma was separated from the cells with a siliconized pipette The cells were washed 3 times with equal volumes of saline and then suspended in saline to original blood volume

Cell suspensions were incubated with thrombin in concentrations of 0 003 0 03 0 3 and 3 NIH units per ml After 20 sec the action of thrombin was interrupted by addition of heparin to a final concentration of 10 I U per ml The cells were separated by centrifugation at 1000 g for 30 min and supernatants were stored at 4° C up to 24 hrs These supernatants will in continuation be referred to as *thrombin supernatants*

Simultaneously with thrombin supernatants *control supernatants* were always made They were prepared in the same way except that heparin was added to the cell suspension prior to thrombin

Assay of potassium and hemoglobin

Potassium was measured by means of a flame photometer (Eppendorf West Germany) Hemoglobin was measured according to Crosby and Furth (1965)

Assay of vasoactivity

The experiments were performed on heparinized mongrel dogs (1000 I.U./kg b.w.) weighing between 10—14 kg Anesthesia was induced by intravenous thiopenthal sodium and maintained with 70 % dinitrogen monoxide in oxygen administred by means of an Engstrom respirator (MIVAB Stockholm Sweden) When necessary further thiopenthal sodium was given intravenously

One femoral artery was exposed and a polyethylene catheter was introduced through a small branch for infusion of the test solutions Infusions were made with polyethylene syringes ... -omagnetic ... catheter ... len) con ... ws in the ... der (Sie-

Results

Vasoactivity obtained from blood cells incubated with thrombin

Supernatants obtained from cell suspensions incubated with thrombin in a concentration of 0 3 NIH units per ml were tested for vasoactivity *(thrombin supernatants)*

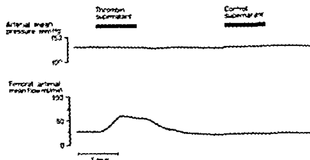


Fig 1 Aortic blood pressure and femoral arterial blood flow during a 1 min infusion of *thrombin supernatant* and *control supernatant* prepared from homologous blood cells Infusion rate 2 ml/min.

Supernatants derived from cell suspensions to which heparin had been added prior to thrombin served as controls *(control supernatants)*

Supernatants from autologous cells were given to 2 dogs in 14 infusions, supernatants from homologous cells were given to 3 dogs in 15 infusions, and supernatants from human cells were given to 2 dogs in 8 infusions The infusion rate of the supernatants varied between 1 and 3 ml per minute to a femoral arterial flow varying between 16 and 99 ml per minute The amount of infused solution rarely exceeded 10 % of the blood flow in the artery The duration of each infusion was 30, 45 or 60 sec

The *thrombin supernatants* always induced immediate increase in blood flow The latter rapidly returned to normal when the infusion was stopped The flow increase following infusion of thrombin supernatants varied between 40 % and 290 % The blood pressure was not affected *Control supernatants* failed to produce changes in blood flow or exerted in some instances only, a small flow increasing effect A typical experiment is shown in Fig 1

The intra arterial concentrations of the test substances were calculated from the infused amount of test substance and blood flow in the artery Vasodilating effect was expressed as per cent flow increase per μl supernatant per ml blood intra intra arterially For *thrombin supernatants* these figures ranged between 1 1 and 5 0 For control supernatants the calculated figures varied between 0 and 0 7 No difference was found between supernatants of autologous homologous or human origin The complete results are listed in Table I

Thus it is obvious that thrombin has the ability to release vasoactive material from the blood cells to the surrounding medium and that this reaction is inhibited by heparin

Thrombin supernatants were then prepared with different concentrations of thrombin The following concentrations were used 3 0 3 0 03 and 0 003 NIH units/ml These supernatants were tested for vasoactivity in 4 dogs

No difference in vasoactivity was seen between supernatants prepared with thrombin in a final concentration of 3 NIH units ml and 0 3 NIH units/ml 0 03 NIH units ml of thrombin still caused release of vasoactive material. When a concentration of 0 003 NIH units ml was used no flow increasing effect of supernatants

TABLE I. Flow changes during intra-arterial infusions of supernatants from autologous, homologous and human blood cell suspensions incubated with thrombin (0.3 NIH/ml). Mean and range values are given

Origin of cells Number of dogs	No of infusions	Thrombin supernatants: Intra-arterial conc µl/ml blood	Flow increase %	Flow increase (%) per µl per ml blood	No of infusions	Control supernatants: Intra-arterial conc µl/ml blood	Flow increase %	Flow increase (%) per µl per ml blood
autologous 2 dogs	7	70 33—190	195 60—390	3.2 1.3—5.0	8	87 37—210	20 0—100	0.1 0—0.7
homologous 3 dogs	15	58 23—94	110 50—320	1.8 1.1—3.1	7	52 28—94	10 0—50	0.2 0—0.5
human 2 dogs	8	31 14—45	60 40—180	3.0 1.6—4.3	3	36 14—48	10 0—20	0.1 0—0.4

could be detected. Thus a concentration of 0.03 NIH units of thrombin/ml is sufficient to release vasoactivity from a cell suspension. The results are summarized in Table II.

Some characteristics of thrombin supernatants

Supernatants from blood cell suspensions incubated with thrombin in concentrations of 0.3 NIH units/ml were used. Potassium and hemoglobin concentrations were measured in 10 canine and 10 human supernatants. As can be seen from Table III, no difference exists between *thrombin supernatants* and *control supernatants*.

Thrombin supernatants were tested for vasoactivity before and after dialysis. After dialysis against an equal amount of saline for two hours, the vasoactivity of the supernatant decreased and vasoactivity was found in the dialysate. After further dialysis against 50 volumes of saline for 24 hrs, the vasoactivity of the supernatant decreased to zero.

Five thrombin supernatants were heated to 100° C for 30 min. Before testing for vasoactivity, the volumes were restored to original level with saline. The vasodilating activity was not affected by this procedure.

Another 5 thrombin supernatants were incubated with an equal volume of autologous plasma at 37° C for 15 min, a procedure which reduced the activity markedly, sometimes even to zero. As controls to the supernatants incubated with plasma, mixtures of supernatants and equal volumes of saline were used.

It is thus concluded that thrombin treatment neither induces hemolysis as estimated by measurement of hemoglobin nor releases significant amounts of potas-

TABLE II Release of vasoactive material after incubation of blood cells with different concentrations of thrombin Results obtained from 4 homologous and 1 autologous supernatant, tested in 4 dogs Mean and range values are given Number of infusions = n

Thrombin conc. in incubation mixture (NIH units/ml)	3 0	0 3	0 03	0 003
Flow increase (%) per µl supernatant per ml blood	2 7 1 0—5 4 n = 8	2 2 1 1—5 0 n = 9	1 9 0 4—5 1 n = 8	 0—0 n = 6

TABLE III Concentration of potassium and hemoglobin in thrombin supernatants and control supernatants (5 of each from human cells and 5 of each from canine cells).

Potassium (meq/l)		Hemoglobin (mg/100 ml)	
Thrombin supernatant	Control supernatant	Thrombin supernatant	Control supernatant
10 supernatants mean 0 298 s d 0 084	10 supernatants mean 0 245 s d 0 078	10 supernatants mean 9 56 s d 6 11	10 supernatants mean 11 59 s d 8 33

sium The vasoactive material released from blood cells during thrombin treatment is dialysable, stable at 100° C for 30 min and is at least partly inactivated by incubation with plasma

Comparison of thrombin supernatants with serotonin, histamine, ATP, ADP, AMP, and adenosine

The circulatory changes produced by thrombin supernatants were compared to those produced by serotonin histamine ATP ADP AMP and adenosine Most of these substances were also exposed to the same treatments as the thrombin supernatants i e heating and plasma incubation Vasoactivity was determined before and after such treatment

Serotonin was infused intra arterially in 5 dogs It induced an immediate increase in femoral arterial flow Concentrations of 10– 20 µg serotonin per ml blood were required to induce the same flow increase as that of thrombin supernatants However a flow increase from serotonin of that magnitude almost always induced a decrease in aortic blood pressure some seconds after the flow increase with a subsequent secondary decrease in femoral arterial flow Furthermore serotonin in contrast to the supernatants usually induced tachyphylaxis (Fig 2)

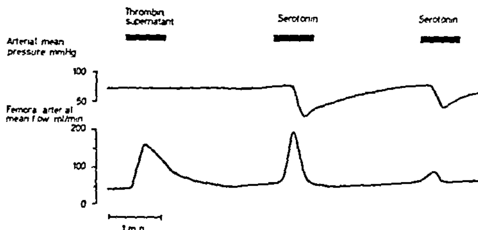


Fig 2 Aortic blood pressure and femoral arterial blood flow during a 45-sec infus thrombin supernatant prepared from autologous cells and of serotonin (0 5 mg/ml) i subsequent infusions Infusion rate of supernatant 3 ml/min of serotonin 2 ml/min

Histamine infusions in concentrations of 2 5—5 μg/ml, resulting in intra ai concentrations of 27—312 ng/ml were made in 3 dogs It caused the same ty reaction as vasoactive supernatants and no alteration in blood pressure cou observed The vasoactive properties of histamine as well as of thrombin s natants remained after exposure to 100° C for 30 min In contrast to thro supernatants incubation with plasma at 37° C for 15 min failed to reduce th tivity of histamine

Adenosine and adenine nucleotides were tested in 5 dogs These substance duced an increase in blood flow similar to that obtained with thrombin superna As calculated from the degree of blood flow increase the vasoactivity of thro supernatants corresponded to 25—75 μg adenosine/ml 100—200 μg AMP/ml 5 μg ADP/ml and 0 5—2 μg ADP/ml The injected solutions gave rise to th lowing intra arterial concentrations for adenosine between 0 8—14 μg/ml for between 2 3—20 μg/ml for ADP between 0 02—0 18 μg/ml and for ATP bet 0 2—1 2 μg/ml In common with thrombin supernatants none of these subst lost their vasoactivity when heated to 100° C for 30 min Incubation with pl however markedly reduced the vasoactivity of ADP and to a lesser degree also of ATP Adenosine and AMP were unaffected by incubation with plasma complete results of the experiments with histamine adenosine and adenine nu tides are listed in Table IV

Treatment of thrombin supernatants with ADP ase and ATP ase (Apyrase)

The vasoactivity of thrombin supernatants was estimated before and after inc tion with Apyrase which is considered to have mainly ADP ase and ATP as tivities To 8 ml of thrombin supernatant were added 1 ml of 0 1 M succinate b pH 6 5, 1 ml of 0 03 M calcium chloride solution and 1 ml of Apyrase solutior

TABLE IV The influence of incubation with autologous plasma at 37° C for 15 min on the vasoactivity of thrombin supernatants, histamine and adenine nucleotides Mean and range values are given Number of infusions = n

Test solution Number of dogs	Flow increase (%) per ng or μl per ml blood	
	Incubation in saline	Incubation in plasma
Thrombin supernatants (3 autologous 1 homologous 1 human) 5 dogs	3 6 2 0—6 2 n = 7	0 82 0—2.1 n = 8
Histamine 2 5—5 μg/ml 3 dogs	1 5 0 69—2 6 n = 8	1 9 1 2—2 9 n = 8
Adenosine 25—100 μg/ml 4 dogs	0 075 0 025—0 158 n = 8	0 073 0 024—0 170 n = 6
AMP 100—200 μg/ml 4 dogs	0 018 0 012—0 028 n = 9	0 022 0 017—0 042 n = 6
ADP 0 5—2 μg/ml 5 dogs	4 0 1.2—6 7 n = 14	0 19 0—1 4 n = 12
ATP 2 5—5 μg/ml 4 dogs	0 84 0 21—1 6 n = 8	0 39 0—0 91 n = 8

mg/ml in succinate buffer) (Krishnan 1946) The mixture was incubated for 1 hr at 30° C and thereafter examined for vasoactivity Thrombin supernatants derived from one batch of autologous and two batches each of homologous and human blood cells were incubated in this way Solutions of ADP (2 μg/ml) ATP (5 μg/ml, AMP (400 μg/ml) and histamine were also incubated for comparison. In control mixtures the Apyrase was replaced by one ml succinate buffer Forty-five infusions were made in two dogs.

Thrombin supernatants irrespective of origin showed markedly decreased or no vasoactivity after the treatment with Apyrase ADP and ATP in the above mentioned concentrations also lost their vasoactivity when thus treated AMP and histamine were completely unaffected

The effects of ADP ATP and thrombin supernatants before and after Apyrase treatment are listed in Table V

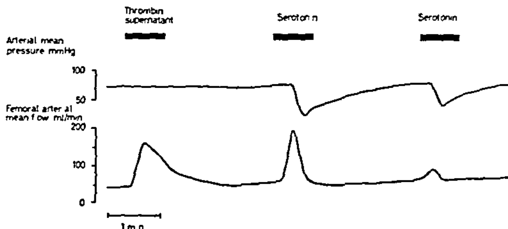


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Histamine infusions in concentrations of 2 5—5 μg/ml, resulting in intra arterial concentrations of 27—312 ng/ml were made in 3 dogs It caused the same type of reaction as vasoactive supernatants, and no alteration in blood pressure could be observed The vasoactive properties of histamine, as well as of thrombin supernatants remained after exposure to 100° C for 30 min In contrast to thrombin supernatants, incubation with plasma at 37° C for 15 min failed to reduce the activity of histamine

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main part of the adenine compounds in the blood is probably found in the red cells (Chambliss *et al* 1952, Rehell Forsander and Raiha 1952) Adenine nucleotides have vasodilating properties (Frohlich 1963, Johnson and Lepley 1966) Serotonin is found in relatively large amount in platelets (Zucker and Borelli 1955, Humphrey and Jaques 1954 Snow *et al* 1955) and it is also liberated when platelets are treated with thrombin (Markwardt and Barthell 1964 Buckingham and Maynert 1964) Also histamine, if present in canine and human platelets might be liberated by thrombin (Humphrey and Jaques 1955) Both serotonin and histamine change the peripheral blood flow (Borst Berglund McGregor 1957)

Serotonin is probably not the vasodilator of importance in the present study In our experiments serotonin always produced generalized circulatory effects when infused intra arterially a phenomenon which was never seen when supernatants were infused A pronounced tachyphylaxis was also observed after infusions of serotonin Repeated infusions of active supernatants seemed to produce no decrease in response Furthermore if the vasodilating effect of thrombin supernatants was due to the content of serotonin it would correspond to at least 0 5 mg/ml of serotonin Such high concentrations of serotonin cannot be obtained from the blood in the incubation mixtures used since the serotonin concentration in platelets—the main source of serotonin—is less than 3 μg per 10^9 platelets (Humphrey and Toh 1954 Zucker and Borrelli 1955 Snow *et al* 1955)

Histamine caused the same type of local vasodilatation as that of thrombin supernatants If histamine was taken into account as the only vasodilator in supernatants its concentration would be 2 5—5 μg/ml This concentration is far too high to emanate from platelets alone since canine and human platelets are considered to contain less than 0 1 μg histamine/10^9 platelets (Humphrey and Jaques 1954) Histamine also differed from thrombin supernatants in not being inactivated by plasma incubation for 15 min.

All the adenine nucleotides had the same flow changing properties as vasoactive supernatants ADP was—on weight basis—found to be 2—5 times as potent as ATP 25—50 times as potent as adenosine and 50—100 times as potent as AMP When concentrations of these substances which produced flow changes of the same magnitude as thrombin supernatants were used it was found that ADP and to some extent also ATP had the same characteristics as thrombin supernatants with regard to heating and plasma incubation

If the vasodilating activity of the supernatants is due to the content of ADP and ATP the concentration might be so low that determinations with current methods would be hazardous In order to find out whether the vasodilating effect of supernatants was due to ADP and ATP they were incubated with Apyrase which is a known specific ADP ase and ATP ase (Krishnan 1946) As expected ADP as well as ATP in relevant concentrations were inactivated by Apyrase AMP and adenosine in concentrations that produced vasodilation in the same range as the ADP and ATP solutions tested were not influenced by Apyrase Histamine was of course unaffected by incubation with Apyrase The vasoactivity of thrombin supernatants

vanished almost entirely after incubation with Apyrase and it thus seems that the main part of the vasoactivity in the supernatants is due to the existence of ADP and/or ATP

The type of blood cells which constitute the source of ADP and ATP in the production of vasoactive material cannot be shown by the present investigation since the different blood cells were not separated However the amounts of ADP which can be released by thrombin from a "normal" number of platelets are according to figures presented by Ireland (1967) large enough to supply the surrounding medium with 0 5—2 μg/ml This amount of ADP does in fact induce circulatory changes of the same magnitude as the vasoactive supernatants The intra arterial concentration of ADP is thus 0 1 μg and less In certain respects our results are in accordance with those of Haustein and Markwardt (1965) who demonstrated that thrombin releases serotonin and ATP from platelets The red cells also contain large amounts of ADP as well as ATP (Chambliss *et al* 1950 Rehell *et al* 1952) and may also be a source of these vasoactive substances. It is not known however whether thrombin can liberate adenine nucleotides from red cells.

We have earlier been able to demonstrate that thrombin when infused intra arterially in a concentration giving just a few hundredths of one NIH unit/ml blood, created a marked decrease in peripheral vascular resistance and that this effect was linked to the enzymatic activity (Delin, Olsson and Teger Nilsson 1967) The concentration of thrombin in the *in vitro* incubations in the present study was of the same magnitude as the intra arterial concentrations in the previous study The vasodilatation during intra arterial infusions of supernatants and thrombin are of the same type i e there is a fast onset of vasodilatation and no influence on blood pressure the latter indicating rapid inactivation of the vasodilating material. It is therefore justified to assume that we are dealing with the same vasodilating agent in the two different studies The peripheral vasodilatation occurring when thrombin is present in the arterial blood may thus be mediated by release of ADP and/or ATP from blood cells ADP has an established role in platelet aggregation *in vitro* (Niewiarowski and Thomas 1966 Gaarder *et al* 1961) and may have importance in hemostasis (Kazer-Glanzmann and Lusher 1962) It is possible that the aggregating ability of thrombin is mediated by release of ADP These experiments indicate that thrombin and ADP also are involved in the regulation of the regional blood flow

The small amounts of thrombin that were required to release vasodilating nucleotides from the blood cells may exist in the blood stream during intravascular coagulation and shock. Since ATP and ADP are such potent vasodilators they may well have significance as agents that reduce the peripheral resistance in many hypotensive states

This work was supported by grants 1?X 1028-01 B68-14X 232?-01 from the Swedish Medical Council.

References

BORN G V R, C I C INGRAM and R S STACEY The proportionality between the amounts of 5 hydroxytryptamine and ATP in blood platelets *J Physiol* (Lond) 1957 *135* 63P—67P

BORST H G, E BERGLUND and J L MC GREGOR The effect of pharmacologic agents on the pulmonary circulation of the dog Studies on epinephrine nor epinephrine 5 hydroxytryptamine acetylcholine histamine and aminophylline *J clin Invest* 1957 *36* 669—675

BUCKINGHAM S and E W MAYNERT The release of 5 hydroxytryptamine, potassium and amino acids from platelets *J Pharmacol exp Ther* 1964 *143* 332—339

CHAMBLISS J R, J DEMMING, K. WELLS, W W KLINE and R W ECKSTEIN, Hemolyzed blood and coronary blood flow *Amer J Physiol* 1950 *163* 545—553

CROSBY W H and F W FURTH Modification of benzedine method for measurement of hemoglobin in plasma and urine *Blood* 1956 *11* 380—383

DELIN A, P OLSSON and A C. TEGER NILSSON Vasodilatation in the canine leg caused by intra arterial infusion of thrombin and tissue thromboplastin *Cardiovascular Research* 1967 *1* 378

FROLICH E D Local effect of adenosine mono-, di and tri phosphate vessel resistance *Amer J Physiol* 1963 *204* 28—30

GRETTE K Studies on the mechanism of thrombin catalysed reactions on blood platelets *Acta physiol scand* 1962 56 Suppl 195 1—93

GAARDER A, J JONSEN, S LALAND and A HELLEM Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets *Nature* (Lond) 1961 *212* 1544—1547

HAUSTEIN K. O and F MARKWARDT, Untersuchungen uber die Freisetzung Gefasswirksamer stoffe beim Ablauf der Gerinnungs- und Fibrinolyse vorgange im Menschlichen Blut *Acta biol med germ* 1965 *15* 386—392

HUMPHREY J H and R JAQUES T ... ts and polymorphonuclear leucocytes of ... —310

HUMPHREY J H and R JAQUES T ... (serotonin) from platelets by antigen a ... 1955 *128* 9—27

HUMPHREY, J H and C C TOH Absorption of serotonin (5 hydroxytryptamine) and histamine by dog platelets *J Physiol* (Lond) 1954 *124* 300—304

IRELAND D M Effect of thrombin in radioactive nucleotides of human washed platelets *Biochem J* 1967 *105* 857—867

JOHNSON W D and D LEPLEY Effect of adenine nucleotides on regional flow *Amer J Surg* 1966 *111* 825—829

KRISHNAN P S Studies on Apyrase II Some properties of potatoe apyrase *Arch Biochem* 1946 *20* 272—283

KAZER GLANZMAN R. and E F LUSCHER The mechanism of platelet aggregation in relation to hemostasis *Thrombos Diathes haemorrh* (Stuttg) 1962 *7* 480—490

MARKWARDT F and W BARTHEL Untersuchungen uber die Freisetsung von Serotonin aus Blutplattchen durch Trombin *Naunyn-Schmiedeberg's Arch exp Path Pharmak* 1964 *249* 176—184

NIEWIAROWSKY S and D P THOMAS Platelet aggregation by ADP and thrombin *Nature* (Lond) 1966 *212* 1544—1547

OLSSON P, J SWEDENBORG and A. C TEGER NILSSON Vasodilatation and hypotension of thrombin in dogs Role of fibrinogen plasma kinins and blood cells *Cardiovascular Research* In press

REHELL B, O FORSANDER and C E RAIHA A micromethod for enzymic determination of ATP in blood *Scand J clin Lab Inv* 1952 *4* 211—215

SNOW P J D, J E LENNARD-JONES, G CURZON and R. S STACEY Humoral effects of metastasizing carcinoid tumours *Lancet* 1955 2 1004—1009

ZUCKER M B and J BORRELLI Relationship of some blood clotting factors to serotonin release from washed platelets *J appl Physiol* 1955 *7* 432—442

Acta physiol. scand. 1969. 76. 148—158
From the Nobel Institute for Neurophysiology, Karolinska Institutet, Stockholm Sweden

Intercostal and Cerebellar Influences on Efferent Phrenic Activity in the Decerebrate Cat

By

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Received 15 August 1968

Abstract

Decima E E and C von Euler *Intercostal and cerebellar influences on efferent phrenic activity in the decerebrate cat* Acta physiol scand 1969 *76* 148—158

The intercostal to-phrenic reflex connections, previously studied only in spinal preparations have now been further analyzed in decerebrate cats and rabbits The early excitatory part of the response was of the same nature as the corresponding response component in spinal preparations The subsequent inhibitory phase was due both to post firing depression as in the spinal state and to active inhibition The responses in decerebrate animals showed marked respiratory variations with almost complete abolishment of the excitatory component in the end expiratory phase In the decerebrate preparation the responses from the different portions of the diaphragm showed qualitative differences which disappeared after spinalization Further signs of differentiated intercostal to phrenic relat ons were obtained by stimulating different intercostal nerves Stimulation of the anterior lobe of cerebellum exerted pronounced effects both on the respiratory phrenic activity—inhibition sometimes followed by post tetanic facilitation—and on the intercostal to-phrenic reflex—removing the inhibition in the end-expiratory phase Some of the features of the synaptic organization of the phrenic nucleus are discussed

In previous papers (Decima Euler and Thoden 1967 1968, Decima and Euler 1968) it was shown that the paucity of muscle spindles in the diaphragm and the corresponding lack of autogenetic facilitation of the phrenic motoneurones (*cf* Corda, Euler and Lennerstrand 1965, also for further references) is, to some extent, made up for as it were by proprioceptive influences from the lower intercostal muscles Evidence was thus presented for a mechanism of self facilitation of the phrenic motoneurones whereby a contraction of the diaphragm by its mechanical action on the lower rib cage elicted an intercostal to-phrenic reflex discharge

A more complex organization of the phrenic motoneurone pool than has commonly been assumed was suggested by the finding that the phrenic motoneurones showed considerable individual differences in their responses to variations in the afferent input from the different intercostal nerves This prompted further studies

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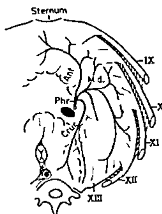


Fig 1 Schematic drawing of right hemidiaphragm, seen from the pleural side, showing its sternal, costal and crural portions and their relations to the main phrenic branches The relations betwen the costal part of the diaphragm and the ribs IX—XII are also indicated

concerning the functional organization of the phrenic motonucleus with special regard to 1) possible differences in the reflex relations between the different inter costal nerves and the main portions of the diaphragm and 2) possible influences of cerebellar stimulation

Whereas our previous papers, cited above, were concerned with intercostal to-phrenic relations in spinal animals, the experiments of the present study were performed on decerebrate preparations since it was soon realized that supraspinal structures exerted some discriminating effects on the reflexes from the different intercostal nerves

Although it had been reported previously (Meulders, Massion and Colle 1960), that cerebellar stimulation would not exert any effect on the diapragm, in contrast to the intercostal muscles (*cf* Corda, Euler and Lennerstrand 1966) it was decided worth while to reinvestigate the matter using also the intercostal-to-phrenic reflex as an index Both inhibitory and facilitatory effects have been obtained in response to cerebellar stimulation

Methods

The experiments were done in adult cats and rabbits decerebrated (midcollicular) under ether anesthesia. Eighteen cats and five rabbits were used The lower external and internal inter costal nerves (VIII—XII) were dissected cut distally and mounted for stimulation (see De [illegible]

discharges were recorded from the C5 phrenic root in the neck as described elsewhere (De cima *et al* 1968) 2) Electromyographic recording of the activity in different parts of the diaphragm was accomplished by means of indwelling electrodes (Basmajian 1961) in the sternal costal and crural parts of both hemidiaphragms 3) Topographical differences in out put from different parts of the phrenic pool was also studied by recording from the main terminal branches of the phrenic nerve To get access to the diaphragmatic branches inside the thorax a large right thoracotomy usually with resection of the 9th or 10th ribs was per formed. The three main phrenic branches (see Fig 1 were then individualized in the lateral side of the inferior vena cava and carefully separated from it They were then dissected free from the surrounding muscular tissue severed distally and mounted for recording The active

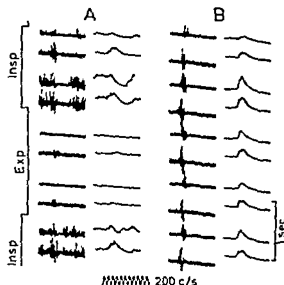


Fig 2 From the decerebrate cat under Flaxedil and artificial respiration Integrated and directly recorded phrenic responses to suprathreshold shocks to the 10th internal intercostal nerve A in the decerebrate state and B after high cervical spinalization with stimulating and recording conditions remaining the same except that stimulus strength was 2 db higher in A than in B To the left in A the phases of the respiratory cycle are marked Upper records in each pair are obtained from the anterior phrenic branch and the lower records in each pair are from the middle phrenic branch Records of the right columns in both A and B are the integrated responses otherwise the same as those of the left columns

lengths of the nerve branches used for recording (10—15 mm) were covered with vaseline Gauze embedded with warm mineral oil was then placed around the recording region covering all exposed tissues (lung diaphragm and part of mediastinum) In this series of experiments the animals were paralyzed with gallamine (Flaxedil®, May & Barker)

Results

he intercostal-to phrenic response in the decerebrate cat

The intercostal to phrenic reflex could be elicited not only after high spinalization as described in preceding papers (Decima *et al* 1967 1968 Decima and Euler 1968) but was easily obtained also in the decerebrate preparations of both cat and rabbit

The general character of the early excitatory part of the phrenic response was very similar to that described for the spinal preparations Some important dissimilitaries were encountered however The response in the decerebrate animal waxed and waned with the animal s own respiratory cycles it generally became greatly depressed or even completely abolished during the last part of the expiratory phase and assumed its maximal size during inspiration These respiratory fluctuations in responsiveness are clearly seen in Fig 2 A and in Fig 5 *A* and *C* In the decerebrate preparation the phrenic response did not usually reach as great a magnitude as in the spinal preparation (*cf* Fig 2 *A* and *B*) However the thresholds for the phrenic response, when elicited in inspiration or in the early part of the expiratory phase were roughly the same as the thresholds obtained in the spinal preparation and the threshold differences between external and internal intercostal nerves were similar in the two types of preparations There were no significant differences in the latencies of the early excitatory phase of the responses in the spinal and the decerebrate animals Nor could any latency change be observed when a decerebrate cat or rabbit

was acutely spinalized (*cf* Fig 2 *A* and *B*) It thus seems justified to assume that the early excitatory part of the response in the decerebrate preparation was of the same nature as that obtained in the spinal animal

Judged against the background of the respiratory impulse activity (inspiratory) in the efferent phrenic fibres, the early volley was generally followed by a period of strong inhibition lasting for almost 15 msec As reported in a preceding paper (Decima *et al* 1968) the early volley of the response in the spinal preparation was followed by a phase of depression which could be observed against the background of the spontaneous' tonic phrenic activity An inhibitory response without a preceding phase of excitation was not observed in the spinal animal In the decerebrate preparation, however, the inhibitory phase of the response appeared to be fairly independent of the foregoing excitation The relative proportions between the early excitatory and the later inhibitory parts of the response were dependent *i a* on which of the intercostal nerves was stimulated A pure inhibitory response without any noticeable preceding excitatory effect could, for example, be obtained by stimulation of the 8th internal intercostal nerve, whereas in the spinal cat stimulation of this nerve always provoked an initial excitation In the decerebrate preparation the inhibitory phase of the response often appeared at one and the same latency independent of whether it was superseded by an early volley or not This can be seen in Fig 2 *A* The inhibitory part of the response was usually of shorter duration in the decerebrate preparation than in the spinal one and was sometimes followed by a late excitatory discharge The latter was never seen without a preceding inhibition the size of these late volleys, however, was always bigger the longer the duration of the preceding inhibition The second inhibitory phase of the response in the decerebrate animal was probably of dual nature a depression corresponding to the response in the spinal animal (Decima *et al* 1968, Decima and Euler 1968) and an active inhibition The relative proportions between the excitatory and inhibitory parts of the responses seem to be dependent on influences from supraspinal structures

Reflex activation of the different parts of the diaphragm

The question whether the phrenic motoneurones innervating the different areas of the diaphragm have different reflex responsiveness has been approached by recording separately from the three main phrenic branches supplying the anterior costal the middle costal and the crural parts of the hemidiaphragm as shown in the diagram of Fig 1

The typical difference between the responses of the anterior costal branch and the branch innervating the crura has already been shown in Fig 2 *A* In this figure the upper record in each pair of sweep tracings was obtained from the anterior costal branch The records from this branch showed a powerful inhibition of the response when it was triggered to fall in the period of inspiration with no or only a small preceding excitatory response component On the other hand the response from the crural branch consisted of a dominating initial discharge followed by a relatively

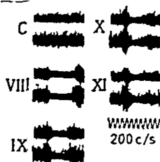


Fig 3 From a decerebrate cat under Flaxedil and artificial respiration To show differences in responses from the crural (upper records in each pair) and the middle costal branches (lower records) of the right phrenic nerve to short tetanic burst stimuli to the internal intercostal nerves VIII—XI Each record is composed of 30 superimposed frames Stimulus strength 4 db above threshold For further explanation see text

weak inhibition The responses from the middle costal branch generally showed an intermediate pattern as illustrated in Fig 2 by the lower records in each pair

In order to establish whether these differences in response obtained from the different phrenic branches were dependent on supraspinal influences, high cervical spinalization was performed on four cats after the decerebrate pattern of response had been obtained These experiments all showed that after spinalization no significant differences in response from the three phrenic branches could be observed The stimulating conditions were kept completely unaltered throughout this part of the experiment

Further differences in the output activity from different parts of the phrenic motoneurone pool were observed when the input was varied by stimulating different intercostal nerves In Fig 3 superimposed records were obtained simultaneously from the crural branches (upper tracings in each pair) and from the middle costal branches (lower tracings) of the phrenic nerve in response to successive stimulation of the internal intercostal nerves 8—11 the records denoted *C* serving as the controls The response to stimulation of the 8th nerve contrasted markedly to those produced by stimulation of the other nerves in that it consisted of an altogheter dominating inhibition preceded by a very weak or no excitatory phase Stimulation of the internal intercostal nerves 9 10 and 11 changed the emphasis of the responses in both the crural and the middle costal leads towards a stronger initial excitation and a decreasing phase of inhibition The responses obtained from the anterior costal branches usually showed a complete absence of an early volley whereas in this branch a prominent inhibitory phase was always obtained to stimulation of the internal intercostal nerves Stimulation of any of the external intercostal nerves 8—10 produced an early excitation only in the branch innervating the crural portion of the diaphragm but not in the branches for the middle and the anterior costal portions, no clear differences were obtained with regard to which of the external intercostal nerves 8—10 was stimulated From the external nerves (8—12) some inhibition was always elicited in all the three leads considerably less powerful however when compared with the effects elicited from the internal nerves

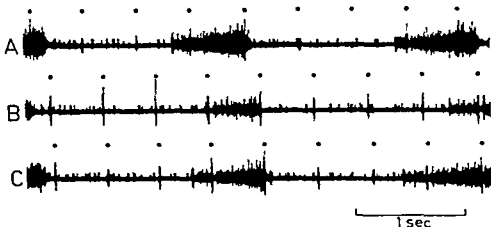


Fig 4 From a decerebrate cat under Flaxedil and artificial respiration To show differential effects of cerebellar stimulation on inspiratory phrenic discharge and on phrenic responses to single shocks to external intercostal XI The intercostal stimuli are marked with dots *A* control before cerebellar stimulation *B* during and *C* immediately after tetanic stimulation of the cerebellar cortex at a contralateral paravermian site on lobulus IV Stimulus rate 190/sec Both vagal nerves were intact so that the inspiratory discharge was kept in phase with each stroke of the pump For further explanations see text

Passive movements of the trunk

A series of experiments was performed on five cats to study whether, in response to passive movements of the lower thoracic and lumbar region of the trunk any effects on the electromyographic activity of different parts of the diaphragm could be detected Dorsal and lateral flexions as well as rotations of about 60° round the vertical axis were the passive movements applied Different effects were thereby usually recorded from the different portions of the diaphragm suggesting the occurrence of a differentiated reflex activation of the different parts of the diaphragm Most of the results however were not repeatable from one experiment to another The only consistent results of these experiments were obtained in response to dorsal flexion of the back which regularly resulted in inhibition of both costal and crural portions of the diaphragm on both sides

Effects of cerebellar stimulation on respiratory and reflex phrenic activity

The intercostal to phrenic relations and the results indicating supraspinal influence on this reflex seemed to offer a new approach to the question on a possible cerebellar control of phrenic activity Tetanic stimulation of the lobulus IV and V of anterior cerebellum usually caused marked inhibitory effects on the inspiratory activity of the phrenic motoneurones This effect was most pronounced on the ipsilateral side Facilitation of the inspiratory phrenic activity was never obtained in direct response to tetanic stimulation of the cortex of the anterior lobe neither when contralateral nor when ipsilateral vermian or paravermian loci were tested As a post tetanic

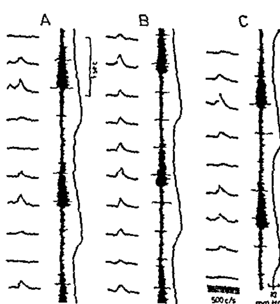


Fig 5 From a decerebrate cat under Flaxedil and artificial respiration To show selective effects of cerebellar stimulation on the intercostal-to-phrenic reflex response without influencing the inspiratory phrenic activity Sweep records of the integrated responses to single shocks to the whole 11th intercostal nerve The continuous records show the directly recorded phrenic activity and the intracheal pressure Both vagal nerves are intact so that the inspiratory discharge was kept in phase with the stroke of the pump For further explanations see text

effect however a slight facilitation of the respiratory phrenic activity was often elicited from paravermian sites both ipsi and contralateral to the side of the phrenic recording

The effects of cerebellar stimulation on the intercostal-to-phrenic reflex followed a quite different pattern Fig 4 illustrates this effect In these experiments the decerebrate cats were fully paralyzed by Flaxedil and the ventilation was adjusted so as to give a good spontaneous rhythmic inspiratory phrenic discharge Since both vagus nerves were left intact in these experiments the animal s respiratory rhythm became locked to that of the pump by the inflation reflex of Breuer (1868 Record *A* of Fig 4 serves as the control and shows both the spontaneous inspiratory activity and the responses to intercostal stimuli in the C5 phrenic root The almost complete depression of the phrenic responses to intercostal stimulation towards the end of the expiratory pause is clearly seen Record *B* of Fig 4 is obtained during prolonged tetanic stimulation of the cortex of lob IV of the anterior cerebellum at an ipsilateral vermian site It illustrates both of two different effects of cerebellar stimulation 1) the marked inhibition of the inspiratory discharge driven by the medullary respiratory mechanisms and 2) the augmentation of the reflex responses to intercostal stimulation especially of those whoch were triggered to fall in the expiratory phase and which thus were strongly inhibited before the beginning of the cerebellar stimulation The latter effect thus resulted in a marked reduction of the respiratory variations of the response This effect is clearly seen in Fig 5

The augmentation of the responses falling in the expiratory phase has also been evoked selectively from cerebellar loci from which little (as in the case of Fig 5) or no effects on the respiratory discharge could be elicited or from which only post

tetanic facilitation of the respiratory discharge was obtained From other stimulus sites the inhibition of the respiratory discharge could be selectively elicited with little effect on the intercostal to-phrenic reflex volley No systematic distribution of the loci yielding the different responses could be recognized they seemed to be intermingled with each other

Discussion

The demonstration in the present paper that the intercostal to-phrenic reflex is readily obtained not only in the spinal cat but also in the decerebrate preparation suggests that this reflex is of functional significance

The early excitatory response seems to all accounts to be of the same nature in both spinal and decerebrate preparations The subsequent inhibitory phases in the decerebrate animals appear to be of dual origin post firing depression, as in the spinal preparations and an active inhibitory mechanism The reasons for this conclusion has already been discussed above in the Results

The fact that the phrenic reflex volley was usually found to be smaller in the decerebrate preparation than in the spinal animal especially in the late expiratory phase suggests that the reflex is under inhibitory influence from supraspinal structures Supraspinal inhibition of thoracic reflexes have been described by Downman and Hussain (1958) Euler and Fritts (1963) and more recently by Alderson and Downman (1966) In the lumbo-sacral segments where supraspinal effects have been studied more extensively, it has been found that the interneurones mediating reflexes elicited by group Ib and II afferents are under tonic inhibition from medullary reticular structures especially from the inhibitory region of Magoun and Rhines (1946 *cf* Eccles and Lundberg 1959) These are the afferent fibre groups which seem to be responsible for the intercostal to-phrenic reflex (Decima *et al* 1968 Decima and Euler 1968) In the present work it has been shown that the phrenic reflex responses to different intercostal nerves which in the spinal cat were all qualitatively similar often showed significant differences in the decerebrate preparation. This suggests that descending influences from supraspinal structures both inhibitory and facilitatory in function are balanced in such a way that selective effects on the reflexes from different intercostal nerves are achieved (*cf* Eccles and Lundberg 1959 Holmqvist and Lundberg 1959 1961) Similarly Alderson and Downman (1966) found that the descending influence from supraspinal structures affected differently the intercostal responses to splanchnic and to intercostal stimulation

The marked respiratory fluctuations of the phrenic response as contrasted to the fairly uniform size of the inter intercostal reflex response (Alderson and Downman 1966) may possibly be explained in part by the respiratory dominance of the diaphragm over the intercostals which is typical for the decerebrate cat (Adrian 1933 Bronk and Ferguson 1935 With relatively little inspiratory activity in the thoracic motonuclei the reciprocal inhibition in the expiratory phase would also be small (*cf* Eklund Euler and Rutkowski 1964

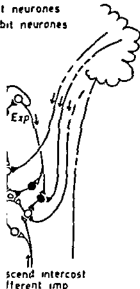


Fig 6 A schematic representation of some features of the synaptic arrangement of the phrenic motoneurones which, in an oversimplified way would explain some of the present results on cerebellar stimulation In this hypothetical diagrams inhibitory functions has only been represented by postsynaptic mechanisms Insp and exp represent the reciprocally occurring inspiratory and expiratory activity descending from the respiratory mechanisms in medulla The pathways from cerebellum are broken to indicate that they might be interrupted by brainstem relays

ohrenic response of the decerebrate preparation was usually smaller than that gh spinalization The reduction of the responses which fell in the phase of *ion might be explained partly on the basis of occlusion However, the re-* that fell in the early phase of expiration before the active expiratory inhibi the inspiratory neurones sets in, was also of smaller magnitude in the de-e preparation than after spinalization This suggests that interneurones ng the intercostal-phrenic reflex like those engaged in the inter-intercostal are under supraspinal inhibitory influence acting during both phases of re-n The strong inhibition of the phrenic reflex during the late part of expira-*ild, however, be almost completely overcome by stimulation of the* anterior cerebellum

erebellar influence on the phrenic motoneurones, like that on the intercostals, viously been denied (*cf* Meulders *et al* 1960) The demonstration of such e therefore constitutes important part of the resent work The influences from the anterior lobe of cerebellum on the phrenic motoneurones appear to iated by two separate pathways one mediating inhibitory effects on the in-y activity and the other executing facilitatory or disinhibitory actions on the tal-to phrenic reflex and thereby outbalancing or removing the active in occurring in the late part of the expiratory phase The latter effect proved be tightly linked to the first mentioned inhibition of the inspiratory phrenic ge and could therefore not be exerted on the final common path Fig 6 rizes in an hypothetical diagram some features of the synaptic connections

previous paper (Decima and Euler 1968) the individual phrenic moto-es were shown to respond quite differently to various intercostal stimuli This ken as a suggestion that the phrenic motoneurone pool is not the simple

homogenous motor relay commonly thought of The effects of cerebellar stimulation as well as the differences in response from the three phrenic branches to stimulation of the lower internal intercostal nerves reported in the present paper indicate furthermore a fairly differentiated synaptic organization of the phrenic motoneurone pool It has been interesting to note that the signs of a functional differentiation of the phrenic nucleus was only revealed in a state close to normal respiratory activity The most attractive aspect of studying respiratory neural activity is the possibility of analyzing a motor system in a fairly normal mode of operation In view of the present results it would be of interest to investigate in detail whether by cerebellar stimulation some sharper contrasts and a more selective differentiation in the relations between trunk wall muscles and the different portions of the diaphragm could be brought in

The functional significance of the intercostal to-phrenic relations are far from clear Although there is little evidence for a true postural function of the diaphragm it does not seem unlikely, however, that for the respiratory function the different portions of the diaphragm would need some co-ordination with the postural movements of the trunk Whether, for instance, the increased contractions of the hemidiaphragm on the lower side in lateral position (*cf e g* Birath 1952, Bergan 1957, also for further references) is due to reflex adjustments or merely due to the fact that the diaphragm on that side is more stretched and therefore exert greater force, is a problem of considerable clinical interest which, however, still remains to be solved

This work was supported by the Swedish Medical Research Council (Project No 14X 544 04) E E D had a research fellowship from the Marci Foundation and a travel fellowship from IBRO Unesco

References

ADRIAN E D Afferent impulses in the vagus and their effect on respiration *J Physiol* (Lond) 1933 *79* 332—358

ALDERSON A M and C B B DOWNMAN Supraspinal inhibition of thoracic reflexes of somatic and visceral origin. *Arch ital Biol* 1966 *104* 309—327

BASMAJIAN J V *Muscle alive Their functions revealed by electromyography* Williams and Wilkins Co Baltimore 1961

BERGAN F *The investigation of the relative function of the right and left lung by bronchospirometry* Oslo Univ Press 1957 Greene & Stratton Inc New York & London 1957

BIRATH [illegible] 52 *66* 134—150

BREUER [illegible] Nervus vagus *S ber Akad Wiss Wien* Abth [illegible]

BRONK I [illegible] ntrol of intercostal respiration *Amer J Physio* [illegible]

CORDA M C v EULER and G LENNERSTRAND Proprioceptive innervat on of the diphragm *J Physiol* (Lond) 1965 *178* 161 17

CORDA M C v EULER and G LENNERSTRAND Reflex and cerebellar influences on α and on rhythm c and tonic γ activity in the intercostal muscles *J Physiol* (Lond) 1966 *184* 898—923

DECIMA E E and C v EULER Excitabil ty of phrenic motoneurones to efferent input from lower intercostal nerves in the sp nal at *Acta physiol scand* 1969 *75* 580—591

DECIMA E E C v EULER a d U TI [illegible] Sp nal intercostal phren c reflexes *Nature* Lond 1967 *215* 312—313

DECIMA E E, C v EULER and U THODEN, Intercostal-to-phrenic reflexes in the spinal cat *Acta physiol scand* 1969 *75* 568—579
DOWNMAN, C B B and A HUSSAIN, Spinal tracts and supraspinal centres influencing visceromotor and allied reflexes in cats *J Physiol* (Lond) 1958 *141* 489—499
ECCLES, R M and A LUNDBERG, Supraspinal control of interneurones mediating spinal reflexes *J Physiol* (Lond) 1959 *147* 565—584
EKLUND G, C v EULER and S RUTKOWSKI, Spontaneous and reflex activity of intercostal gamma motoneurones *J Physiol* (Lond) 1964 *171* 139—163
EULER C v and H W FRITTS, Quantitative aspects on respiraory reflexes from lungs and chest wall *Acta physiol scand* 1963 *57* 284—300
HOLMQVIST B and A LUNDBERG Differential supraspinal control of ... nal reflex actions from ... 71
... synaptic actions evoked ... *physiol scand* 1961
MAGOUN H W and K RHINES, An inhibitory mechanism in the bulbar reticular formation *J Neurophysiol* 1946 *9* 165—171
MEULDERS M J MASSION and J COLLE, Influence du lobe anterieur du cervelet sur l'activité tonique et respiratoire des muscles intercostaux *Arch ital Biol* 1960 98 430—440

Acta physiol. scand. 1969. 76 159—171

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Release, Reuptake and Net Uptake of Dopamine, Noradrenaline and Adrenaline in Isolated Sheep Adrenal Medullary Granules

By

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Received 19 August 1968

Abstract

Lishajko, F , *Release, reuptake and net uptake of dopamine, noradrenaline and adrenaline in isolated sheep adrenal medullary granules* Acta physiol scand 1969 *76* 159—171

tonic K phosphate
different rates fol
in granules during
NA) was released
rate DA in con
or DA to 34 min,
owing to reuptake and decreased it for A to 43 min

Addition of ATP Mg 3 mM considerably decreased the release rate of DA as well as that of NA and A presumably owing to inhibition of release and increased reuptake In the presence of ATP Mg the addition of 3×10^{5} M DA further retarded the net release of DA

presence of ATP-Mg This effect is
served in partially depleted granules
retards the release of soluble proteins
concomitantly with catecholamines The rapidly released NA has been localized in the heavy granule fractions

The presence of DA in the sheep adrenal was discovered by Goodall (1951) and later confirmed by Shepherd and West (1953) for bovine adrenals Eade (1958) was able to show that the DA present in the bovine adrenal was bound to a 'large-granule' fraction and amounted to about 1 per cent of the total catecholamines

Extraadrenal DA was first reported by Euler and Lishajko (1957) to occur in bovine lung tissue and by Montagu (1957) in the brain Its presence in specific cells was demonstrated by Falck, Hillarp and Torp (1959) The finding that brain storage particles can take up DA after administration of dopa (Weil Malherbe 1960) made it likely that DA is normally stored in specific granules

Carlsson, Hillarp and Waldeck (1962, 1963) and Kirshner (1962 a b) have

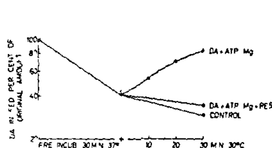


Fig 4

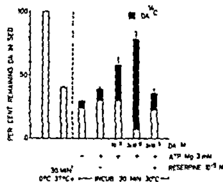


Fig 5

Fig 4 Sheep adrenal medullary granules, net DA uptake after partial depletion in isotonic K-phosphate, pH 7 5 by preincubation 30 min at 37° C, continued incubation for 30 min at 30 C. Addition of 3×10^{-5} M DA, 3 mM ATP-Mg (●—●), 3×10^{-5} M DA, 3 mM ATP-Mg and 5×10^{-6} M reserpine (■—■), control (no addition) ○—○ Ordinate per cent DA in sediment of original amount Abscissa incubation time in min Each point is the mean of 2 expts

Fig 5 Sheep adrenal medullary granules, incubated as in Fig 4 with labelled ^{14}C-DA and unlabelled DA Columns represent remaining DA in sediment after second incubation in per cent of original amount Filled area, incorporated ^{14}C-DA, calculated from SA_{SED}/SA_{M} Additions as indicated ± SEM n = 7

that in the presence of DA in a concentration of 3×10^{-6} M in the incubation medium (no addition in control) the incorporation of ^{14}C-DA corresponds to 14 per cent of the total remaining DA in the sediment With 10^{-5} M and 3×10^{-5} M of DA the incorporation of ^{14}C DA corresponds to 25 and 47 per cent respectively

While ATP-Mg alone raised the reuptake of ^{14}C-DA to 28 per cent of the remaining DA in the sediment (total concentration of DA in the medium 3×10^{-6} M), addition of DA at 10^{-5} or 3×10^{-5} M together with ATP-Mg to the incubation fluid further increased the incorporation of ^{14}C-DA and non-labelled DA to 58 respectively 84 per cent of the remaining DA in the granules The higher amount of DA in the sediment (above the control at +4° C) after incubation with 3×10^{-5} M DA and ATP-Mg may indicate the presence of partially depleted granules The same phenomenon is sometimes observed for NA in the nerve granules (Euler and Lishajko 1967) Reserpine at a concentration of 10^{-5} M decreases the reuptake of ^{14}C-DA as well as that of nonlabelled DA

Net uptake of DA in partially depleted granules

As seen in Fig 4 preincubation of the granules for 30 min at 37° C decreases the DA content to about 40 per cent of the original amount Subsequent incubation for 30 min at 30° C with DA 10^{-5} M and 3 mM ATP-Mg raised the DA content to 58 per cent of the original amount and to 83 per cent when 3×10^{-5} M DA was added to the incubation fluid Practically complete restoration of the DA content in the granules was observed in 40 min at 30° C Net uptake of DA was also stimulated by ADP-Mg although to a lower extent than with ATP-

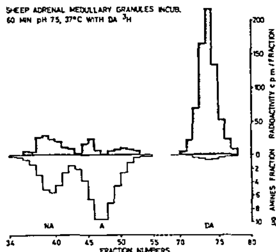


Fig. 6. Sheep adrenal medullary granules incubated in isotonic K phosphate pH 7.5 for 60 min at 37° C, with addition of 3×10^{-4} M DA, 3 mM ATP-Mg and labelled DA-³H, sp. act. 3.5 C/mmole (conc. 1.7×10^{-8} M). Chromatographic separation on Amberlite CG 120. Fractions read in 285 mμ (act.) and 335 mμ (fluor.) respectively. Abscissa: fraction numbers. Column length 15 cm.

Net incorporation of ¹⁴C-DA was studied in a similar way as in the reuptake experiments. After partial depletion of the granules ¹⁴C-DA and non-labelled DA were added together with ATP-Mg 3 mM before the second incubation period (Fig. 5). No net incorporation of DA was observed when DA was present in a concentration of 0.2—0.8×10^{-5} M in the medium. During the same time DA in the control without ATP-Mg fell from 40 to 29 per cent of the original amount in 30 min at 30° C.

Addition of 10^{-5} or 3×10^{-5} M DA together with ¹⁴C-DA and ATP-Mg 3 mM increased the incorporation of radioactive DA to 48 and 80 per cent respectively of the remaining DA in the sediment, so that after the end of the second incubation the granules have largely restored the DA content which was lost during the first incubation. Reserpine at 10^{-5} M inhibits the net uptake of DA into the granules.

In order to establish that the labelled DA incorporated into the granules existed as such the granule suspension was incubated with labelled and nonlabelled DA together with ATP-Mg and the extract of the sediment used for chromatographic separation (Fig. 6). The figure shows that most of the incorporated labelled DA is in the DA containing fractions (71—78).

Spontaneous release of NA and A in comparison with DA

Fig. 7 shows that the NA release pattern is characteristically different from that of A and DA. The approximately straight line obtained for A with semilogarithmic representation up to 90 min at 37° C corresponds to a $t/2 = 49$ min. The DA shows a similar exponential release up to about 15 min while at further incubation this is moderately slowed down presumably due to increasing concentration of DA in the incubation medium and reuptake. The NA in the granules falls rapidly in the beginning of the incubation as previously reported by Oka *et al.* (1966). Thus after 5 min at 37° C the granules have lost about 30 per cent of NA into the medium

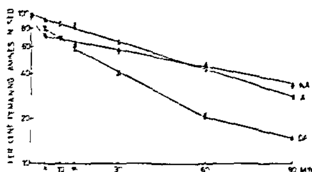


Fig. 7 Sheep adrenal medullary granules incubated in isotonic K phosphate, pH 7 5, 37 C. Ordinate per cent remaining DA NA and A of original amount (control at +4 C) Abscissa time in min ± SEM, n = 7

After this period the NA release rate is even slower than that for A (t/2 = 77 min) Between 5 min and 90 min incubation, 1 6 times more A than NA is released or 39 per cent NA and 64 per cent A

Reuptake and net uptake of NA and A in the presence of ATP-Mg

a. *Undepleted granules*

Incubation of the granules with ATP-Mg markedly lowers the net release of NA and A from the granules presumably owing to inhibition of release and reuptake (Fig 8

After addition of ^{14}C-labelled dl NA and dl-A together with 3 mM ATP-Mg the incorporation of ^{14}C-amines into the granules during 60 min incubation at 37° C measured and expressed as the specific activity ratio of NA and A in sediment supernatant (SA$_{(s)}$/SA$_{(s)}$) From Fig 8 it can be seen that in the control (no addition) the incorporation of ^{14}C-NA corresponds to 5 per cent of the total remaining NA in the sediment respectively 11 per cent for the A After addition of 3 mM ATP-Mg together with ^{14}C-labelled amines the incorporation of ^{14}C-NA corresponds to 10 per cent respectively 16 per cent for the A of the total remaining amines in the sediment The incorporated labelled NA and A thus corresponds only to about 30 and 40 per cent respectively of the amine content in excess of the control amount

Addition of 3 × 10⁻⁴ M DA together with ATP Mg causes no significant change of the NA and A in the granules in 60 min at 37° C over and above that caused by ATP-Mg alone while DA is taken up almost to the original amount (not shown in Fig 8) (^{14}C-A not added in last column)

Reserpine at 1 × 10⁻⁴ M markedly diminished the ATP Mg stimulated reuptake of DA and NA, but to a somewhat lesser extent that of A

b *Partially depleted granules*

Granules which have been partially depleted by incubation for 30 min at 37 C show no net uptake of NA or A during further incubation with ATP Mg 30 min at 30° C when the amine concentrations in the medium were about 2—3 × 10⁻⁶ M NA and 4—7 × 10⁻⁵ M A at the end of the incubation period (Table I) In this respect the NA and A granules differ markedly from the DA granules

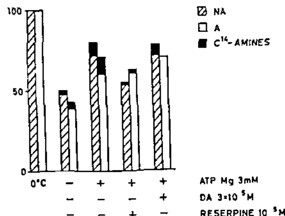


Fig 8

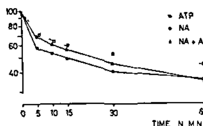


Fig 9

Fig 8 Sheep adrenal medullary granules incubated in isotonic K phosphate pH 7 5 60 min at 37° C Ordinate per cent remaining amines in sediment Additions as indicated Free amines in medium 10⁻⁶ M NA and 10⁻⁶ M A at the end of incubation

Fig 9 Sheep adrenal medullary granules incubated in K phosphate, pH 7 5 at 37° C Ordinate per cent remaining amines and ATP of original amount in sediment ATP (□—□) NA (○—○), and NA+A (△—△) in sediment after incubation Abscissa time in min

Rapid release of NA

In view of the rapid initial release of NA from sheep adrenal medullary granules it seemed of interest to study whether granules from other species show the same behavior The result of these studies are presented in Table II which gives the average half times for NA, A and DA in isolated granules at 37° C

Since NA is released at different rates, the half times for this amine are given both for the rapid initial phase (0—5 min) and for the second slower phase (10—60 min) The half times for A and DA remain approximately constant during the time period 0—60 min From the table it can be seen that NA shows remarkably

TABLE I Sheep adrenal medullary granules partially depleted by incubation for 30 min at 37° C Further incubation for 30 min at 30 C with addition of 3 mM ATP Mg (free amines in medium 2—3×10⁻⁶ M NA and 4—7×10⁻⁶ M A) The table shows remaining NA and A in sediment in per cent of original amount (control at +4° C) ± SEM n = 8

Preincubation 30 min 37 C		Further incubation for 30 min at 30° C			
		No addition		Addition 3 mM ATP Mg	
NA	A	NA	A	NA	A
55 ± 3	61 9 ± 1 5	43 5 ± 2 8	51 4 ± 1 8	44 8 ± 3	57 4 ± 1 7

TABLE II Half time in minutes during spontaneous release of DA NA and A from adrenal medullary granules in various species Incubation period 0—60 min Half times for NA release given for initial rapid period (0—5 min) and slow period (10—60 min)

Species	Half time (t/2) at 37° C			
	NA 0—5 min	NA 10—60 min	A 0—60 min	DA
Bovine	21	107	83	24
Sheep	11	77	49	22
Sheep (fetal 83 days)	35	98	60	21
Goat	17	112	36	18
Horse	15	48	47	
Pig	8 5	65	36	
Cat	11	45	33	
Dog	69	76	49	
Monkey (Synomolges	70	62	30	
Chromaffin cell tumors	85	85	52	18
Rabbit			21	
Guinea pig			46	
Rat	6	38	12	
Fowl	4 5	40	37	

different release rates during the two phases in all species containing NA with the exception of the dog the monkey (*Synomolges*) and human phaeochromocytoma in which the release rate for NA is uniform

The sheep adrenal storage granules release 30 per cent of their NA in approximately 5 min in the initial phase which represents a release rate approaching that for adrenergic nerve granules in an amine free medium (Euler and Lishajko 1967)

In order to examine whether the time lapse between the death of the animal and the start of the experiment was of importance for the rapid NA release phenomenon the adrenals were removed within 2 12 and 22 min after the death of the animal (exsanguination) No significant difference in release rate of either NA A or DA was observed Fig 9 also shows that ATP and CA (NA+A) are released at the same rate up to 15 min (the ratio CA/ATP in the sediment in 2 expts was 4 6 and 4 8 respectively) After this time the ratio CA/ATP in granules slowly decreased and after 60 min at 37 C it had fallen to 3 7 Isolated granules from fowl adrenals show a similar decline of CA/ATP ratio from 6 3 (control) to 5 1 in 60 min at 37° C

It is known that in isolated chromaffin granules the release of CA is accompanied by a release of ATP soluble protein and some ions as calcium (Ca^{++}) and magnesium (Mg^{++}) To investigate the release during the initial stage of incubation of

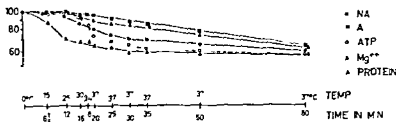


Fig 10 Bovine adrenal medullary granules incubated in isotonic K phosphate, pH 7 5 Temperature raised from + 4° C to 37° C in 20 min Ordinate per cent remaining of original amounts in sediment Abscissa temperature and time intervals Each point is the mean of 2 expts

these components, bovine adrenal medullary granules were incubated at gradually increasing temperatures, from ice water temperature up to 37° C in 20 min (Fig 10) The figure shows that 15 per cent of the Mg⁺⁺ had been released when the temperature had increased to 15° C (see time in Fig 10) When temperature had reached 2o° C, the Mg release was 30 per cent of original amount At 37° the Mg release was 35 per cent compared to 27 per cent of ATP release NA release was noticeable at 15° C, and amounted to 20 per cent at 37° C The granules release 10 per cent of soluble protein when the temperature reaches 37° C Soluble proteins (which constitute 85—90 per cent of total granule protein) and adrenaline are released exponentially at all time and temperature intervals The rapid spontaneous release of Mg⁺⁺ and ATP may play a role in the rapid fall of NA

In order to test the possibility of separating a specific fraction of the NA granules with a high release rate, bovine adrenal medullary granules were subjected to differential centrifugation for 5—30 min (Fig 11) The sediment of each fraction was resuspended and incubated at 37° C The figure shows rapid spontaneous release of NA in all three fractions sedimenting at $1000 \times g$ and $2000 \times g$ containing 70 per cent of the total amines in the sediment, while the $3000 \times g$ fraction shows a slower NA release This fraction presumably is mixed with a previous fraction and corresponds to about 20 per cent of the total catecholamine in sediment

The fractions obtained at 4 5, 9 and 50×1000 g show a release of NA at an identical and slow rate These fractions correspond to about 15—20 per cent of the sum total of CA in all fractions As can be seen the release of A, in contrast to that of NA is constant suggesting a "homogenous pool of A" The NA to A propor-

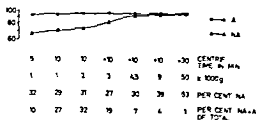


Fig 11 Bovine adrenal medullary granules incubated 5 min in K phosphate pH 7 5 37° C after differential centrifugation Ordinate per cent remaining \ \ and \ of original amount in sediment Centrifugation time, gravity per cent \ \ per fraction and per cent \ \ + \ of original amount Each point is the mean of 3 expts

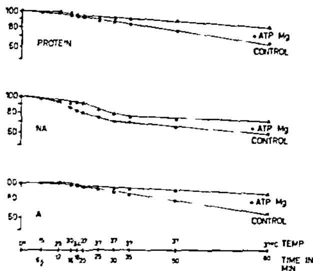


Fig 12 Bovine adrenal medullary granules incubated as in Fig 10 Soluble protein, NA and A in sediment after incubation with 3 mM ATP Mg (▲—▲) and without addition (●—●) Temperature and time intervals indicated Each point is the mean o 2 expts

tions in different fractions are quite similar except in the 9 and 50×1000 *g* fractions which show higher figures

Addition of ATP Mg 3 mM to the medium of bovine adrenal medullary granules (Fig 12) using the same experimental conditions as previously in Fig 10 inhibits the release of soluble protein as well as of NA and A at all intervals of time and temperature This appears to indicate that soluble proteins and CA leave the storage concomitantly also in the presence of added ATP Mg

Fig 13 shows the relationship between CA and soluble protein released in the supernatant after different incubation times (up to 120 min) The experimental conditions are the same as in Fig 10 Fig 13 shows a close relationship between CA and soluble proteins even after addition of ATP Mg Of particular interest is the observation that Segontin (prenylamine) causes a parallel release of CA and soluble protein in all the three concentrations tested At 3×10^{-5} M Segontin releases practically all of the CA as well as of the soluble protein and ATP in 60 min at 37° C

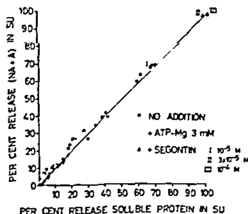


Fig 13 Bovine adrenal medullary granules incubated in isotonic K phosphate pH 7 5 The figure shows the correlation between the release of NA+A (ordinate) and soluble proteins (abscissa) without additions (○) with 3 mM ATP Mg (●) and Segontin (prenylamine (▲)

Discussion

The results with granules from the sheep and bovine adrenal medulla indicate that DA is bound to the granules in addition to NA and A and that DA is released at a considerable higher rate with t/2 about 22 min at the prevailing low DA concentration in the medium as compared to about 49 min for A NA showed a rapid initial rate with t/2 about 11 min and a much slower release after 10 min with a t/2 of 77 min (Fig 7) confirming the observations of Oka *et al* (1966) However, in difference to the Japanese workers we have found a constant slow release of NA after the initial rapid fall

The results suggest either that NA granules with different release rates exist or that NA is given off at different rats from two storage sites within the same granules or that a fraction is rapidly released from a uniform pool in the initial stage

The finding that most of the rapid NA release is bound to the heavy granules fraction (Fig 11) speaks in favour of the first possibility and may indicate that this fraction corresponds to specific NA granules

Blaschko Hagen and Welch (1955) observed that 15 to 25 per cent of the NA and A in medullary granules were readily released Hillarp (1960) assumed different amine pools in bovine adrenal medullary granules suggesting that the amines are more readily released from ATP deficient granules No distinction between A and NA was made It might be that the 30—50 per cent readily released NA in the sheep adrenal medullary granules correspond to a special type of granules

The exponential release of A shows a constant rate in all incubation periods and in all fractions (Fig 10 and 11), suggesting that adrenal medullary granules in different species store A in a homogenous pool and are different from DA and NA granules In the dog and monkey and in human phaeochromocytoma (see Table II) NA and A seem to be stored in a homogenous pool

The rapid release rate of NA in the beginning of incubation might conceivably be due to a deficit of ATP in the granules owing to postmortal discharge of ATP but not of an equivalent amount of amine However since no significant difference in release rate of the amines or CA/ATP ratio was found when sheep adrenals were removed within 2 to 22 min after the death of the animals this explanation appears less probable

The DA granules also differ from the others in other respects Addition of 3×10^{-4} M DA to the incubation medium strongly inhibits the net release of DA from the granules owing to reuptake while the corresponding addition of NA or A to the medium does not alter the normal release rate of these amines However in the presence of ATP Mg 3 mM reuptake of all amines is stimulated (Fig 8) The concentration of free NA and A in medium were 1×10^{-4} M at the end of the incubation period

The effects of ATP Mg on reuptake of NA and A correspond to about 30—40 per cent of the amount in excess of the control while 60—70 per cent is due to inhibition of release (Fig 8 as judged by the incorporation of labelled amines

The finding of Carlsson Hillarp and Waldeck (1962 1963) and of Kirshner (1962a b) that uptake of CA in adrenal medullary granules is stimulated by ATP Mg has thus been confirmed The effect of ATP on the uptake suggests that it may supply energy for some process, possibly involving active transport of amine into the granules The active reuptake may occur as long the granule can use the ATP energy Spontaneous reuptake of NA and A without addition of ATP Mg occurs only to a small extent

It is also conceivable that the ATP-Mg added to the incubation medium inhibits the release from a Mg ATP protein or other complex (*cf* Fig 11) This is supported by the finding that addition of ATP Mg *into the medium retards release* of soluble protein from the granules

Another explanation seems to be that the amines leaving or about to leave the granules are more efficiently reincorporated into the granules in the presence of ATP Mg in the medium (Euler and Lishajko 1967) The reason why DA is re incorporated spontaneously into the granules is unknown but the same phe *nomenon is observed for NA in nerve granules (Euler and Lishajko 1962 1967)* The DA granules seem to be similar to the nerve granules in this as in several other respects.

The characteristic inhibition of the ATP Mg stimulated reuptake of DA on ad dition of reserpine in concentrations of 1×10^{-5} M to the medium also holds true for NA but less so for A (Fig 8), suggesting some membrane action on the rapidly eleasing NA granules and little action on the "insensitive slow releasing A granules) The lack of net uptake of NA and A in contrast to DA in granules after partial depletion Fig 5) might be explained by a loss of a Mg ATP protein or complex binding mechanism concomitantly with the amines (Hillarp 1958) In partially depleted granules the net uptake of amine into the granules apparently only occurs if a mechanism is available for binding of the amines Still the amines in the partially depleted granules may exchange with the amine in the incubation medium

The failing net uptake after partial depletion may explain the slow repletion of amines into the adrenal medulla after insulin and hypoglycemia (Hokfelt 1951, Udenfriend *et al* 1953) On the other hand Bygdeman, Euler and Hokfelt (1960) found that synthesis of CA can still take place at a rapid rate during insulin hypoglycemia, but most of the newly synthesized amines is secreted into the blood

This suggests that insulin *in vivo* as other drugs *in vivo* and *in vitro* deplete NA and A together with ATP and soluble protein (as in spontaneous release *in vitro*) In this case the empty or partially depleted granule can not incorporate amines to the normal content until new formation of the adrenal medullary granules occurs

The research reported in this document has been sponsored in part by the AIR FORCE EOAR-68-0009 through the European Air Force and by the Swedish Medical

References

BLASCHKO H P HAGEN and A D WELCH Observations on the intracellular granules of the adrenal medulla *J Physiol* (Lond) 1955 *129* 27—49

BYGDEMAN S U S v EULER and B HOKFELT Resynthesis of adrenaline in the rabbit s adrenal medulla during insulin induced hypoglycemia *Acta physiol scand* 1960 *49* 21—28

CARLSSON A N A HILLARP and B WALDECK A Mg^{++} ATP dependent storage mechanism in the amine granules of the adrenal medulla *Med exp* (Basel) 1962 *6* 47—53

CARLSSON A N A HILLARP and B WALDECK Analysis of the Mg^{++} ATP dependent storage mechanism in the amine granules of the adrenal medulla *Acta physiol scand* 1963 *59* Suppl 215

CARLSSON A and B WALDECK A fluorimetric method for the determination of dopamine (3 hydroxytyramine) *Acta physiol scand* 1958 *44* 293—298

EADE N R The distribution of the catecholamines in homogenates of the bovine adrenal medulla. *J Physiol* (Lond) 1958 *141* 183—192

EULER U S v and F LISHAJKO Dopamine in mammalian lung and spleen. *Acta physiol pharmacol neerl* 1957 *6* 295—303

EULER U S v and F LISHAJKO Improved technique for the fluorimetric estimation of cate cholamines *Acta physiol scand* 1961 *51* 348—355

EULER U S v and F LISHAJKO Release and uptake of catecholamines in nerve storage granules in relation to adrenergic neuro-transmission *Arch int pharmacodyn* 1962 *139* 256—280

EULER U S v and F LISHAJKO Reuptake and net uptake of noradrenaline in adrenerg c nerve granules with a note on the affinity for l and d isomers *Acta physiol scand* 1967 *71* 151—162

FALCK B N A HILLARP and A TORP A new type of chromaffin cells probably storing dopamine *Nature* (Lond) 1959 *183* 267—268

GOODALL McC Studies of adrenaline and noradrenaline in mammal an heart and supra renals *Acta physiol scand* 1951 *24* Suppl 85

HILLARP N A The release of catecholamines from the amine containing granules of the adrenal medulla *Acta physiol scand* 1958 *43* 292—30?

HILLARP N A Different pools of catecholamines stored in the adrenal medulla. *Acta physiol scand* 1960 *50* 8—2?

HAGGENDAL J On the use of strong exchange resins for determinations of small amounts of catecholamines *Scand J clin Lab Invest* 1962 *14* 537—544

HOKFELT B Noradrenaline and adrenaline in mammalian tissues. Distribution under normal and pathological conditions with special reference to the endocrine system *Acta physiol scand* 1951 *25* Suppl 93

KIRSHNER N Uptake of catecholamines by a particulate fraction of the adrenal medulla *Science* 1962 a *135* 107—108

KIRSHNER N [illegible] he adrenal medulla *J biol C*[illegible]

LISHAJKO F [illegible] ranules in the sheep adrenal [illegible]

LOWRY O H N J ROSENBROUGH A L FARR and R J RANDALL Protein measurement with the folin phenol reagent *J biol Chem* 1951 *193* 265—275

MONTAGU K A Catechol compounds in rat tissues and in brains of d fferent an mals *Nature* (Lond) 1957 *180* 244—245

OKA M T OHUCHI H YOSHIDA and R IMAIZUMI Selective release of noradrenaline and adrenaline from isolated adrenal medullary granules. *Life Sci* 1966 *5* 433—438

SHEPHERD D M and G B WEST Hydroxytyramine and the renal medulla. *J Physiol* (Lond) 1953 *120* 15—19

STREHLER B L and J R TOTTER Determination of ATP and related compounds Firefly luminescence and other methods In "*Methods of Biochemical Analysis* Ed D Glick 1954 *1* 341—356

UDENFRIEND S J R COOPER, C T CLARK and J E BAER Rate of turnover of epinephrine in the adrenal med lla. *Science* 1953 *117* 663—665

WEIL MALHERBE H Synthesis of catecholam nes in the depleted bra n In "Adrenergic Mechanisms Ciba Foundation S mp Ed G E W Wolstenholme and M O Connor London Churchill 1960 544 554

Acta physiol. scand. 1969. 76. 172—181

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Metabolism of ^{14}C-Histamine in Domestic Animals

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Received 20 August 1968

Abstract

Eliassen K. A. *Metabolism of ^{14}C-histamine in domestic animals I Goat* Acta physiol scand 1969 76 172—181

It has been suggested that increased *in vivo* formation of histamine might be of importance in the pathogenesis of some diseases in ruminants. However little is known about the degradation of iv injected histamine in these species. In the present study isotope dilution technique and paper chromatography were used to examine urinary ^{14}C-metabolites subsequent to iv injection of ^{14}C-histamine to the goat. During the first week after administration 80—90 per cent of the activity was found in urine and about 1 per cent in feces. Known metabolites accounted for 91—108 per cent of the urinary radioactivity Oxidative deamination of histamine to imidazoleacetic acid and its riboside seemed to be the only pathway of quantitative importance for histamine degradation in the goat. Paper chromatography revealed that histaminol (4(5) imidazolyl-ethane-2-ol) is a metabolite of histamine in the goat accounting for about 2 % of the urinary radioactivity ^{14}C-histaminol is formed when liver or kidney homogenates are incubated with ^{14}C-histamine

The main features of the metabolism of parenterally administered ^{14}C-histamine are well established in man and most laboratory animals methylation of ring nitrogen and oxidative deamination are quantitatively the most important pathways for histamine degradation. In domestic animals however little is known about ^{14}C-histamine degradation Sjaastad (1967 a) reported oxidative deamination to be more important for histamine inactivation in sheep than in other species previously examined.

It has been suggested that increased *in vivo* formations of histamine might be of importance in the pathogenesis of some diseases in ruminants (Åkerblom 1934 Dougherty 1942 Dain Neal and Dougherty 1955 and Nilsson 1963) It would be of interest to examine the histamine metabolism under pathological conditions but to evaluate histamine metabolism in diseases it is first necessary to obtain information on the metabolism of histamine in healthy animals. A study of ^{14}C-histamine degradation in healthy animals was therefore undertaken

A preliminary communication of part of this work has been presented elsewhere (Eliassen 1967)

Abbreviations

Hi	histamine
AcHi	N acethylhistamine
MeHi	methylhistamine
HiOH	imidazoleetane 2-ol (histaminol)
ImAA	imidazoleacetic acid
ImAA R	imidazoleacetic acid ribosid
MeImAA	methylimidazoleacetic acid

Materials and methods

Histamine (ring $^{14}C_2$) dihydrochloride (30 5 mC/mM) was purchased from the Radiochemical Centre Amersham England 1 4 methylhistamine dipicrate (1 methyl-4(β aminoethyl) imidazole dipicrate) was synthesized in the Department of Chemistry, University of Oslo The 1 4 methylhistamine dipicrate was converted to the dihydrochloride by passage through a Dowex® 1 column. The 1 4 MeHi dihydrochloride was crystallized and recrystallized 2—3 times from an ethanol acetone mixture before use

1 5 methylimidazoleacetic acid was a gift from G Granerus, Department of Clinical Physiology, University of Gothenburg Sweden.

The following materials were obtained commercially Imidazoleacetic acid HCl 1 4 methyl imidazoleacetic acid HCl and N acetylhistamine all A grade (Calbiochem Los Angeles U S.A) Histamine dihydrochloride and histamine diphosphate (Sigma Chemical Co, St Louis Missouri, U S A) Dowex® 50 W × 4 (100—200 mesh) and Dowex® 1 × 8 (200—400 mesh) (Fluka Bursh S G Switzerland) Amberlite resin CG50 (100—200 mesh) (British Drug Houses Ltd England) Film for radioautography Industrex® Type D (Kodak, London England) Silica gel G acc to Stahl (E Merck, Darmstadt, Germany) High resolution® buffer pH 8 9, (LKB Stockholm Sweden)

Six healthy female goats of Norwegian breed housed in metabolism cages were used for the experiments The diet consisted of pelleted concentrates hay and kohlrabi Water *ad libitum* was allowed The cages permitted separate quantitative collection of urine and feces To the collection flasks was added sufficient HCl to bring the pH of the urine under 2 Feces were sampled every 24 hrs and were then suspended in N HCl

Biological estimation of histamine in urine

Free histamine was determined as previously described (Eliassen and Sjaastad 1968) Histamine was adsorbed on a cation exchange resin and the eluate tested on guinea pig ileum The acetone treated urine was boiled with 10 N HCl for 1 1/2 hrs to convert conjugated histamine to free histamine

Injection of ^{14}C labelled histamine

Just prior to the injection the ^{14}C-histamine was purified on a Dowex 50 W column (30 × 4 mm according to Kahlson Rosengren and Thunberg (1963) Histamine was eluated from the column by 0 5 ml 10 N HCl followed by two portions of 1 ml of water The eluate was neutralized with NaOH and adjusted to a final volume of 50 ml by adding water and saline in such proportions that the resultant solution was close to isotonic Of this solution 0 5 ml was used for counting The remaining 49 5 ml containing about 24 μC was injected into the jugular vein

Determination of radioactivity in urine and feces

All radioactive samples were counted on a Beckman Lowbeta II flowcounter (background 1 3 2 5 cpm) From 10 to 100 μl of undiluted urine were plated for counting Feces was homogenized in N HCl and boiled under reflux for 10 min After centrifugation 100 μl of the supernatant were plated for counting Self absorption was measured by counting urines to which internal standards of ^{14}C-histamine had been added and the results were corrected on this basis Further correction was made for background activity

Isotope dilution assays

Urine collected during the first 24 hours after ^{14}C Hi administration was subjected to isotope dilution assays for histamine and its metabolites The methods used were developed by Schayer and described in parts by Schayer and others

In the present study some modifications have been introduced and the analytical procedure was as follows Generally the carrier of each specific substance to be identified was added to urine aliquots whereupon the substances were extracted

After treatment with activated charcoal, the metabolites were crystallized as the picrate or the pipsyl derivative. The samples were then repeatedly crystallized and treated with activated charcoal. When the radioactivity was constant throughout three recrystallizations purity was considered to have been achieved. All samples of crystals were counted on standard plates (2.5 cm in diameter) at infinite thickness.

Histamine (Carrier 66.4 mg Hi dihydrochloride). Histamine was extracted with 40 ml n-butanol by shaking for 30 min in a Griffin flask shaker. Before extraction the urine (20 ml) was made strongly alkaline with 1 ml 10 N NaOH and saturated with anhydrous Na_2SO_4 (8 g). Histamine was re-extracted from the butanol layer with 15 ml N HCl. The HCl layer was removed and evaporated to dryness on a water bath. The residue was dissolved in 3 ml distilled water and treated with activated charcoal. To the filtrate 220 mg p-iodophenyl sulphonyl chloride (pipsyl chloride) dissolved in 4 ml acetone (Thunberg 1965) and 0.5 g $NaHCO_3$ was added. The mixture was shaken by hand for about 20 minutes. Water was then gradually added until no more crystals were formed. After filtering the histamine pipsyl crystals were rinsed with 25 % (w/v) icecold ethanol and plated for counting.

1,4-Methylhistamine (Carrier 104.5 mg 1,4-MeHi dihydrochloride corresponding to 1.5× the histamine carrier). From the urine (20—30 ml diluted to 30 ml) previously made strongly alkaline with 2 ml 10 N NaOH and saturated with anhydrous Na_2SO_4 (6 g) 1,4-MeHi was extracted three times with 20 ml chloroform. To the pooled extracts 3 ml HCl saturated abs. ethanol was added before evaporation to dryness. The residue was dissolved in 3 ml water whereupon picric acid (150 mg) dissolved in 4 ml abs. ethanol was added and then the volume was reduced by heat to about 3 ml. Crystals of methylhistamine picrate were formed when the mixture was cooled and a glass rod was rubbed against the bottle wall. After filtration the crystals were rinsed with icecold abs. ethanol as well as ether. The alcoholic suspension of the crystals was then transferred to counting plates.

Free imidazoleacetic acid and 1,4-methylimidazoleacetic acid (Carrier 196.0 mg of ImAA hydrochloride and 196.5 mg of 1,4-MeImAA hydrochloride corresponding to twice the Hi carrier). The imidazole compounds were adsorbed from the acidified urine (2—5 ml urine and 1 ml HCl up to 30 ml with water) on a cation exchange column (Dowex® 50 W 60× 10 mm). The resin had been thoroughly washed with 0.1 N HCl and water. The acids were eluted from the column with 75 ml N NH_4OH. The basic eluate was collected and treated with activated charcoal before filtration. The filtrate was then evaporated to dryness and the residue was dissolved in 3 ml water. For ImAA 450 mg pipsylchloride dissolved in 4 ml acetone and 250 mg $NaHCO_3$ were added. After shaking the mixture for 30 min acetic acid was added to give a pH about 5 and then water was added slowly until no more crystals were formed. As for pipsylhistamine the ImAA pipsylcompound was rinsed with 25 % (w/v) icecold ethanol. For 1,4-MeImAA 310 mg picric acid dissolved in 5 ml abs. ethanol was added and crystals were formed as for 1,4-MeHi.

Imidazoleacetic acid riboside (Carrier 196.0 mg ImAA hydrochloride). The urine was evaporated to dryness and the residue was dissolved in 3 ml HCl. By hydrolysis with HCl at 150° C in sealed tubes for 5 hrs ImAA-R will completely split into ImAA and ribose provided that the pH is below 1.5 after hydrolysis. It is noteworthy that pH increases to a variable degree during hydrolysis probably due to the presence of different amounts of organic materials in the samples. With the quantities of urine used in the present experiment 3 ml of N HCl was sufficient to bring the pH below 1.5. Total ImAA was determined in the hydrolysed urine (1—3 ml) in the same way as described for free ImAA. ImAA-R was then calculated as the difference between total ImAA and free ImAA.

Conjugated histamine (Carrier 83.0 mg N-acetylhistamine corresponding to 1.5× the Hi carrier). Conjugated histamine was determined as the increase in free Hi by boiling the urine (20 ml) with 10 N HCl under reflux for 1.5 hrs.

Calculation in isotope dilution assays. To calculate the relative contents of each metabolite in the urine the ratio between cpm obtained for standard solutions of [14]C-histamine at zero thickness (counted on plates of 6.4 cm diameter) and cpm obtained at infinite thickness (counted on plates of 2.5 cm diameter) was used. With 66.4 mg Hi dihydrochloride as carrier the ratio was 30.3 when Hi was crystallized as pipsylchloride (258.3 mg) and 2.6 when Hi was crystallized as picrate (205.4 mg).

Example. The urine at zero thickness gave 10 760 cpm/ml. The activity of Hi-pipsyl chloride crystals (based on 20 ml urine and 66.4 mg Hi dihydrochloride carrier) was 202.5 cpm after reaching constant activity. Hence Hi constitutes 202.5 · 30.3 · 100/10 760 · 20 = 2.9 % of the total radioactivity in the urine.

Since 196.0 mg ImAA hydrochloride and 196.5 mg MeImAA hydrochloride corresponds to twice the Hi carrier, the counts found for the pipsylchloride of ImAA and the picrate of MeImAA must be doubled. Likewise since 104.5 mg MeHi hydrochloride and 83.0 mg

N acetylhistamine correspond to $1.5 \times$ the H_1 carrier the corresponding counts must be multiplied by 1 5

Paper chromatography

Minor non identified metabolites of histamine cannot easily be detected by isotope dilution technique Therefore aliquots of 100 to 250 μl urine were subjected to two-dimensional descending paper chromatography

Whatman paper no 1 and the following solvent systems were used

1 n-Butanol — acetic acid — water (4 1 1, v/v/v)
2 n Butanol saturated with 20 % NH_3
3 Phenol — chloroform — ethanol — NH_3 (14 14 10 2 w/v/v/v) (White 1963)
4 Isopropanol — pyridine — water (2 2 1 v/v/v)

For detection of ^{14}C-H_1 metabolites, X ray films were exposed to the chromatograms for 4 to 8 weeks Semiquantitation of the metabolites was obtained by counting the radioactive areas identified by help of carrier substances The carrier substances were made visible by spraying the chromatograms with one or several of the following reagents

1) diazotized p-nitroanaline followed by 10 % (w/v) Na_2CO_3 2) 1 % (w/v) p-nitrobenzenediazonium fluoborate in acetone (Shifrine and Zweig 1964), 3) 1 % (w/v) iodine in ethanol 4) 0.3 % (w/v) ninhydrin in acetone — n butanol (1 1)

The two first mentioned spraying reagents could not be used on chromatograms developed in solvents containing phenol For visualization of MeH_1 ninhydrin was superior to the other reagents In order to detect Me

Since ImAA riboside carrier
it to ImAA with N HCl in se
paper chromatography using so

Separation of the histamine
Fig 3 a b

Cellulose acetate electrophoresis An unidentified spot on the paper chromatograms was eluted with water and subjected to paper electrophoresis High resolution® buffer pH 8 9 and 0 4 mA/cm was used Developing time was 20—30 min.

Incubation of kidney and liver tissue Kidney and liver tissue were prepared and incubated as previously described by Sjaastad (1967 b) The tissue homogenates were incubated for 1 5 hrs at 37° C The incubation was stopped by heating the incubation mixture to 100° C After cooling in icebath 1 ml N HCl was added to bring the pH under 2

The incubation mixture was centrifuged at $3000 \times g$ for 10 min and 100—200 μl of the supernatant was subjected to paper chromatography

Results

Urinary excretion of histamine

By biological technique 21 and 14 urines from 6 goats were examined for free and conjugated H_1 respectively In all urines from one goat the free H_1 could not be determined by the method used since the urine prosseed antihistamine-like activity In urines from the other goats free H_1 could not be detected in 4 cases Judged from the sensitivity of the guinea pig ileum used on these occasions urinary histamine in quantities less than about 1 5 μg/24 hrs could not be quantitated When mean values are used the urinary excretion of free and conjugated H_1 were $1 9 \pm 0 4$ (S D) and $8 2 \pm 2 6$ (S D) μg H_1 base/24 hrs respectively

Excretion of ^{14}C radioactivity

Urine

The recoveries of radioactivity in the urine during the first week for experiments no 1—4 were as follows 85 89 80 and 91 % these values being measured with an accuracy of ± 3 %

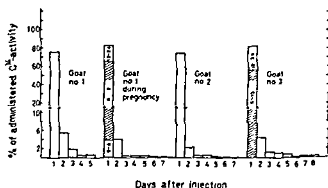


Fig 1 Urinary excretion of radioactivity the first week after injection of ^{14}C labelled histamine

Fig 1 demonstrates the urinary excretion of radioactivity during the first week after i v injection of ^{14}C-labelled Hi. Most of the radioactivity was excreted during the first 24 hrs. After the fifth day, only traces were detected.

Feces

During the first week after administration from 0 69 to 1 37 % of injected radioactivity was recovered in feces. Fig 2 shows the fecal excretion of radioactivity.

Identification of ^{14}C-histamine metabolites in the urine

The results are shown in Table I. Since the goat in exp no 2 was in the fourth month of pregnancy the results from this experiment were not included when mean values were calculated.

For Hi the activity of the pipsyl chloride compound remained constant from the fourth to the eighth crystallization onwards, for conjugated Hi from the third to the seventh crystallization, for total ImAA from the second to the fifth crystallization and for free ImAA from the second to the seventh crystallization.

The activity of the 1 4 MeHi picrat remained constant from the second to the seventh crystallization. The activity of the 1 4 MeImAA picrat fell continuously and was in most instances absent after the fifth crystallization. At this point sufficient quantities of the substance were not left to obtain infinite thickness afer recrystallization.

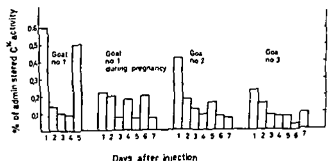


Fig 2 Fecal excretion of radioactivity the first week after i v injection of ^{14}C labelled histamine

TABLE I Catabolism of ^{14}C-histamine in goat
Urinary excretion of ^{14}C activity unchanged ^{14}C histamine and its main metabolites subsequent to intravenous injection of ^{14}C-histamine The values represent the means of duplicate analyses except for MeHi where only one analysis was performed on each urine

Experiment No	Goat No	Radio-activity in the first 24 hr urine (% of amount injected)	Histamine metabolites in % of total ^{14}C in the first 24 hr urine							
			Histamine	1 4 Methyl histamine	Imidazolacetic acid Free	Imidazolacetic acid Riboside	Acetyl histamine	Methyl imidazole acetic acid**	Histaminol **	Sum of metabolites
1	A	77	2 3	0 02	42	49	0	13	1 9	108
2	A*	84	2 8	0 27	30	40	0	15	2 4	90
3	B	76	1 6	0 16	48	33	trace[2]	13	1 6	97
4	C	83	1 0	0 10	46	30	trace[2]	12	1 5	91
	Mean	79	1 6	0 09	45	37		13	1 7	98

* Pregnant in the 4th month and not included in the mean values
** Determined by means of paper chromatography [1]

However the radioautograms showed a spot corresponding to the 1 4 MeImAA carrier The substance having a R_f corresponding to 1 4 MeImAA was eluted and rechromatographed in the system (no 4) used by Karjala and Turnquest (1955) for separating the 1 4 and the 1 5 isomers of MeImAA Reference 1 4 MeImAA split into 3 spots of which the one with the lowest R_f value showed a mobility nearly identical to that of reference 1 5 MeImAA Radioactivity was only found in this area By thin layer chromatography using the same solvent however reference 1 4 MeImAA gave only one spot (R_f = 0 15) which was clearly separated from the reference 1 5 isomer (R_f = 0 25) Radioactivity was only found in the area of the 1 4 MeImAA spot To the eluate of the radioactive substance having R_f corresponding to 1 4 MeImAA carrier 1 4 MeImAA and picric acid were added Constant radioactivity of the 1 4 MeImAA picrat was obtained from the first crystallization

The Hi pipsyl chloride crystals from hydrolysed urines did not give significantly higher counts than the Hi crystal from untreated ones In accordance with these results the radioautograms of chromatograms developed in solvent systems 1/2 gave no spot corresponding to AcHi An unidentified spot with R_f close to reference AcHi was however observed The unidentified spot accounted for 1 5—2 4 % [1] of the total activity on the chromatograms The unidentified substance was eluted and rechromatographed in 4 different solvent systems The R_f values were close to those of AcHi in all solvent systems They are shown in Table II The unidentified substance and reference HiOH when chromatographed together covered each other The unidentified radioactive substance had the same electrophoretic properties as

[1] Values are not corrected for uncomplete recovery due to the chromatography

TABLE II Chromatographical data for histaminol

Solvent system	Rf	
	HiOH	AcHi
n Butanol acetic acid water (4 1 1, v/v/v)	26 ± 2	32 ± 3
n Butanol saturated with 20 % NH_3	71 ± 6	74 ± 10
Phenol chloroform ethanol NH_3 (14 14 10 2, w/v v/v)	74 ± 4	77 ± 8
Isopropanol pyridine water (2 1 , v/v/v)	72 ± 4	76 ± 4

acetylhistamine in the described celluloseacetate system The substance was not hydrolysable It was extractable with n Butanol from an alkaline urine

By means of two-dimensional paper chromatography (solvent systems 1/3) Hi metabolites in urine from experiment no 2 at different times after i v injection of ^{14}C-histamine were semiquantitatively determined The results are shown in Table III It is evident that the ratio between ImAA-R and ImAA increased with time

Incubation of ^{14}C histamine with liver- and kidney-tissue

Formation of a compound with the same chromatographic properties as the unidentified urinary metabolite assumed to be HiOH, was observed when liver and [illegible] homogenates were incubated aerobically with ^{14}C-Hi In one experiment in [illegible]ch the substrate concentration was high the unidentified substance was formed in quantities sufficient to give a red-violet spot on the chromatograms stained with p-nitroaniline and Na_2CO_3 Fig 3 and 4 show radioautograms of chromatograms of liver tissue incubates on which the HiOH spot can be clearly seen

TABLE III Histamine metabolites in % of excreted radioactivity in urine from experiment no 2, at different times after i v injection of ^{14}C-histamine

	Paper chromatographic determination				Istotope dilution determination
	0—6 hr	6—12 hr	12—24 hr	sum 24 hr	24 hr
Hi	11 4	0 7	0 5	1 2	2 8
MeHi	2 1	0 3	0 3	0 2	0 27
HiOH	7 2	1 2	0 9	2 4	—
ImAA	64 5	35 5	18 3	34 8	30 —
ImAA R	1 8	39 2	70 3	45 2	40 —
MeImAA	8 1	17 2	8 2	15 1	—
Appl. point	5 0	6 1	1 6	1 2	—
Sum	100 1	100 1	100 1	100 1	—

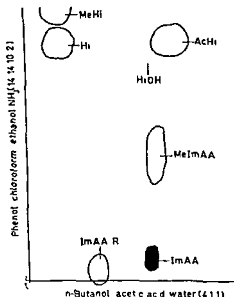


Fig 3

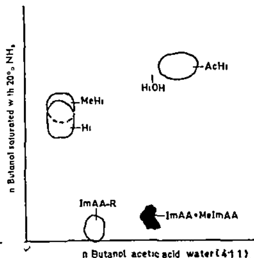


Fig 4

Fig 3 Radioautogram of a typical two-dimensional chromatogram of the extract of liver tissue incubated with ^{14}C histamine n Butanol Acetic acid Water (4 1 1 v/v/v) used as solvent in the first direction and phenol chloroform ethanol NH_3 (14 14 10 2 w/v/v/v) in the second direction

Fig 4 Radioautogram of a typical two dimensional chromatogram of the extract of liver tissue incubated with ^{14}C histamine n Butanol acetic acid water (4 1 1, v/v/v) used as solvent in the first direction and n Butanol saturated with 20 % NH_3 in the second direction

Discussion

In the present study the excretion of radioactivity along with the urine during 24 hrs after i v injection of ^{14}C Hi varied little compared with the large variation of ^{14}C excretion after injection of ^{14}C Hi in man (Nilsson *et al* 1959, Beall and VanArsdel Jr 1960) Experiments nos 2 and 4 demonstrate that about half the injected radioactivity is excreted within 12 hrs However only collection of urine by means of a catheter can give exact data about the rate of urinary excretion of any substance in animals

In the present study part of the radioactivity of the feces collected during the first 24 hrs after injection may be due to contamination with urine Fecal excretion of radioactivity during the first days was low compared with that of the urine From about the sixth day however the quantities excreted by the two mentioned routes of elimination were about of the same order

Generally the pattern of fecal excretion of radioactivity is slower than should be expected from the data on the retention time for food in the digestive tract of ruminants (Balch 1961 Coombe and Kay 1965

To permit valid conclusions with regard to the metabolism of injected ^{14}C Hi from analyses of the metabolites in the urine it is necessary that most of the in

jected radioactivity is excreted by this route In the present experiments this prerequisite seemed to be met since about 80 % of the injected radioactivity could be accounted for in the analysed urine Table I shows that the quantities of each histamine metabolite in goat urine varied little compared with the variation in the metabolic pattern observed in man (Nilsson *et al* 1959)

It has been demonstrated in rat that as much as 70 % of ^{14}C histamine metabolites are excreted as ImAA in the urine (Westling 1958) The present experiments demonstrate that oxidative deamination is still somewhat more important in goats

From direct isotope dilution experiments it was not possible to infer whether the oxidative product of 1 4 MeHi, 1 4-MeImAA was excreted in the urine The present chromatographic data nevertheless indicate that ^{14}C-1 4-MeImAA does occur in considerable amounts in the urine This appears to be confirmed by the fact that the eluate of 1 4-MeImAA from the paper chromatograms gave constant radioactivity from the first crystallization if crystallized with 1 4 MeImAA as carrier

From paper chromatography and crystallization analyses it can be concluded that acetylhistamine or other forms of conjugated histamine, if present, constitute less than 0 5 % of the total radioactivity

It is well established that Hi is oxidatively deaminated to ImAA via imidazole acetaldehyde Evidence of a reductive pathway of Hi metabolism to the corresponding alcohol has been obtained in man and rat (Nakajima and Sano 1964) In normal rat urine they found about 0 5 μg imidazolylethanol/mg creatinine and in healthy man less than 0 1 μg/mg creatinine In the present investigation a compound assumed to be identical with imidazolylethanol (histaminol, HiOH) was found This compound was found on the paper chromatograms and accounted for about 2 % of the urinary radioactivity To obtain further evidence for the identity of this compound the experiments of Nakajima and Sano were repeated with ^{14}C Hi Two untreated rats and two rats given the aldehyde dehydrogenase inhibitor disulfiram® (tetraethylthiuram disulphide) orally were examined In the present study chromatograms of the urine gave a radioactive spot chromatographically identical with that found by analyses of goat urine In nontreated rats the metabolite accounted for about 2 5 % of the excreted ^{14}C activity but in urine from disulfiramtreated rats it accounted for approximately 10 % This appears to support the assumption that the unidentified compound found in goat urine after injection of ^{14}C-Hi is identical to that found by Nakajima and Sano in rat and human urine Incubation experiments showed formation of a HiOH like compound and thereby demonstrated that this urinary metabolite is partly or entirely of endogenous origin The present observations indicate that the conversion of Hi to HiOH in goat and rats represent a regular but a relatively minor metabolic pathway

Analogous pathways for ^{14}C serotonin to ^{14}C 5 hydroxytryptophol have been demonstrated in man (Davis *et al* 1966) and rats (Kveder Iskric and Keglevic 1962)

Since only about 2 % of the i v injected Hi is excreted unchanged it seems that goats are fairly well equipped to metabolize Hi that reaches the systemic circulation

The results reported in the present paper demonstrate that oxidative deamination is the only pathway of quantitative importance for the degradation of Hi in goats The same conclusion was reached for sheep by Sjaastad (1967 a), who found a marked increase in urinary excretion of histamine in aminoguanidine treated animals

Known Hi metabolites accounted for 91—108 % of the total radioactivity of the urine in the present study Technical difficulties may account for the failure to obtain figures closer to 100 % The occurrence of minor quantities of unidentified metabolites however, can not be excluded

This investigation was supported financially by the Agricultural Research Council of Norway
I am grateful to Miss Maj Britt Johansson who generously instructed me in Schayer's isotope dilution technique during a stay at the Clinical Physiological laboratory Lasarettet Lund Sweden (head prof H Westling)
The technical assistance of Miss Tordis Digernes is appreciated

References

AKERBLOM E *Über die Ätiologie und Pathogenese der Futterrehe beim Pferde* Walter de Gruyter & Co Berlin & Leipzig 1934

BALCH C C Movement of digesta through the digestive tract In *Digestive Physiology and Nutrition of the Ruminant* (ed by Lewis D) Butterworths London 1961 23—34

BEALL G N and P P VANARSDEL JR Histamine metabolism in human diseases *J clin Invest* 1960 *39* 676—683

COOMBE J B and R N B KAY Passage of digesta through the intestines of sheep Retention times in the small and large intestines *Brit J Nutr* 1965 *19* 235—238

DAIN J A A L NEAL and R W DOUGHERTY The occurrence of histamine and tyramine
5 *14* 930—935
n of 5 hydroxytrypto
966 *122* 890—893
J Vet Res 1942 *3*
401—402

ELIASSEN K A Histamine metabolites in the goat Report at *Seventh int Congr Biochem* Tokyo 1967

ELIASSEN K A and Ø V SJAASTAD Effects of formaldehyde and formic acid on histamine metabolism in sheep *Acta pharmacol* (Kbh) 1968 *26* 00—00 In press

KAHLSON G E ROSENGREN and R THUNBERG Observations on the inhibition of histamine formation *J Physiol* (Lond) 1963 *169* 467—486

KARJALA S A and B W TURQUEST The characterization of two methyl imidazoleacetic acid as radioactive histamine metabolites *J Amer chem Soc* 1955 *77* 6358—6359

KVEDER S S ISKRIC and D KEGLEVIC 5 hydroxytryptophol a metabolite of 5 hydroxy tryptophane in rats *Biochem J* 1962 *85* 447—449

NAKAJIMA T and I SANO A metabolite of histamine 4 5 imidazoyl etane 2-ol *Biochim biophys Acta* (Amst) 1964 *82* 260—268

NILSSON K S E LINDELL R W SCHAYER and H WESTLING Metabolism of ^{14}C labelled
Clin Sci 1959 *18* 313—319
mental studies of laminitis in cattle *Acta*
the sensitivity of sheep to histamine given
inary excretion of endogenous histamine

SJAASTAD Ø V The in vitro catabolism of histamine by sheep liver tissue *Acta physiol scand* 1967 b *71* 6—16

SHIFRINE M and G ZWEIG Chromatographic separation of histamine and some metabolites on cellulose phosphate paper *J Chromatog* 1964 *14* 297—300

THUNBERG R Personal communication 1965

WESTLING H The difference in the metabolism of injected ^{14}C histamine in male and female rats *Brit J Pharmacol* 1958 *13* 498 500

WHITE T Personal communication 1965

Acta physiol. scand. 1969. 76. 182—190

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pH-Dependence of the Mechanism in the Duodenal Bulb Inhibiting Gastric Acid Responses to Exogenous Gastrin

By

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Received 21 August 1968

Abstract

ANDERSSON, S. and G. NILSSON. *pH-dependence of the mechanism in the duodenal bulb inhibiting gastric acid response to exogenous gastrin.* Acta physiol. scand. 1969. 76. 182—190.

Gastric secretion was stimulated by intravenous infusion of gastrin in Pavlov and Heidenhain pouch dogs provided with isolated pouches of the duodenal bulb. Lowering the intrabulbar pH to pH 1.1—1.3 almost abolished the acid response to a low dose of gastrin, whereas the inhibition of secretory responses to higher doses was less pronounced. Inhibition of secretory responses to a fixed submaximal dose of gastrin did not occur until the bulbar pH was reduced to 2.0—2.5. Secretory responses to threshold doses of gastrin, on the other hand, were significantly inhibited already at pH 3—4. The results question the concept that very low intraduodenal pH is required to activate the mechanism by which acid in the duodenum inhibits gastric acid secretion.

It is well established that acidification of the duodenal mucosa inhibits gastric acid secretion. From experiments on dogs it has been suggested that the duodenal pH must be reduced below pH 2.5 before inhibition of gastric secretion occurs (Pincus *et al.* 1944, Andersson 1960). Since such low pH-values have not been considered to exist in the duodenum under physiological conditions, the physiological significance of the inhibitory mechanism has been questioned (Pincus *et al.* 1944). However, in 1961 Andersson and Uvnäs suggested that there exists a pH-gradient within the duodenum, pH values being low in the duodenal bulb and higher in the distal portions of the duodenum. If this was so, it seemed reasonable to assume that the postprandial pH in the duodenal bulb could become sufficiently low to activate an inhibitory mechanism. Andersson and Uvnäs (1961) substantiated their hypothesis by showing that acid perfusion of isolated bulbar pouches significantly inhibited secretory responses to a test meal from Pavlov pouches.

Subsequent studies on man (Andersson and Grossman 1965) revealed that the pH of the duodenal bulb was better correlated to the pH of the antrum than to the pH of the postbulbar duodenum. Thus when the pH of the gastric antrum was low

(pH 1—3), the bulbar pH was also low and close to the antral pH. Postbulbar pH was stable and usually within the range pH 6.5—7.5. The existence of a pH gradient within the duodenum was later confirmed by Rhodes and Prestwich (1966). Recent studies on dogs have also demonstrated that the mechanism by which acid in the duodenum inhibits gastric acid secretion is mainly located in the duodenal bulb (Andersson, Nilsson and Uvnäs 1965, 1967).

The conclusion that the duodenal pH has to be reduced below pH 2.5 before the inhibitory mechanism is activated has been based on experiments in which attempts were made to reduce the pH of the whole duodenum (Pincus *et al.* 1944, Andersson 1960). In those studies the infused acid became diluted with bile from the intermittently emptying gall bladder and with secretions from the pancreas and the duodenal mucosa. Acid solutions were introduced into the proximal portion of the duodenum and were allowed to flow freely down the duodenum. Samples for determination of the pH of the duodenal content were collected from the distal portion of the duodenum. Although large volumes of acid were infused, fluctuations of the pH of the duodenal perfusate occurred. In our opinion such experiments are not suited to elucidate the pH dependence of the inhibitory mechanism. For example, the experimental conditions did not guarantee adequate perfusion of the bulbar mucosa, the pH of the duodenal perfusate varied and samples for determination of the pH of the perfusate were not taken from the bulbar area from which inhibition is elicited, but from the distal portion of the duodenum. In view of these circumstances, adequate information of the threshold level of acidity at which the inhibitory mechanism is activated is still not available.

The present study was undertaken to determine the critical pH level at which the bulbar mechanism can be activated and also to elucidate the correlation between the inhibitory levels of acidity and the intensity of activation of the acid secreting glands. The experiments were performed on Pavlov and Heidenhain pouch dogs provided with isolated pouches of the duodenal bulb. Gastric acid secretion was stimulated by exogenous gastrin.

Methods

Surgical procedures

Seven dogs weighing 13—18 kg were used. Five of them were prepared with mucosal septal pouches (Pavlov type) and two had separated gastric pouches of the Heidenhain type. All dogs were provided with isolated pouches of the duodenal bulb and gastrojejunostomy. The surgical procedures for these preparations have been described elsewhere (Andersson and Uvnäs 1961). Between each operation and before starting the experimental series the dogs were allowed 3—4 weeks for recovery.

Gastric secretory stimulant

In all experiments gastrin was used as secretory stimulant. The gastrin extract in series B was prepared from hog antral mucosa according to the method described by Grossman and Gillespie (1963). For the other series of experiments gastrin was prepared by a simplified mode of extraction using the ion exchange cellulose DEAE, which was introduced for extraction of gastrin by Gregory and Tracy (1964).

1 kg of antral mucosa was cut in small pieces and boiled in 6 l of distilled water for 20 min with continual stirring. After cooling in an ice bath to 20° C, the suspension was filtered

through gauze (5—6 layers) The filtrate was centrifuged for 10 min at 3000 rpm The remaining fat layer was removed from the centrifuge bottles by skimming The supernatant solution was decanted and the precipitate was discarded The supernatant was adjusted to pH 5 0 with 0 1 N HCl 2 5 g of ion exchange powder (DEAE) per litre was added After stirring for 3 hrs the solution was centrifuged for 10 min at 3000 rpm The supernatant was discarded and the DEAE—floc was washed twice, each time with 1 l of distilled water The gastrin was eluted the DEAE—floc 4 times each time with 225 ml of 0 1 N NaOH for 10 min with continuous stirring After each elution the solution was centrifuged for 10 min at 3000 rpm The supernatants were pooled and adjusted to pH 7 0 with 12 N and 0 1 N HCl Finally the volume was adjusted to 1 l so that 1 ml of the extract corresponded 1 g of antral mucosa extracted The extract was kept at $-20°$ C Its potency was stable for at least one year which is the longest time a batch has been stored The gastrin extract was free from histamine like activity when assayed on guinea pig ileum

Doses of gastrin are expressed as wet weight of antral mucosa extracted Each dog received gastrin from the same batch throughout the tests

Experimental procedures

Each experiment started in the morning after the dogs had been fasted for 18—24 hrs The basal gastric output was recorded for one hour and the secretory responses were collected in 15 min samples The volume was measured and the acidity was determined by titration with 0 01 N NaOH using phenolphthalein as indicator

Four series of experiments were performed In series A a dose of gastrin producing about 30 *per cent of maximal stimulation was given as a continuous i v infusion by a peristaltic* pump (Harvard Apparatus Co Dover Mass, USA) The secretory response had usually reached a plateau within 2—2 1/2 hrs The duodenal bulb was then perfused during one hour with 0 1 N hydrocloric acid or buffer solutions of different pH values After the perfusion period the secretion was followed for a further 1 1/2 hrs The degree of secretory inhibition was calculated from the mean acid output of the last two 15 min periods of perfusion and is expressed as a percentage of the mean 15 min acid output during the hour (control hour) immediately preceding the hour of bulbar perfusion

In series B experiments were performed as in series A The doses of gastrin were selected to produce approximately 30 60 and 90—100 per cent of the maximal response to gastrin The effect of bulbar perfusion with 0 1 N HCl was then determined at each secretory level

In series C the secretory responses to graded doses of gastrin were determined during ontinuous perfusion of the duodenal bulb with buffer solutions of pH 3 and pH 7 Each do e gastrin was given for 1 hr and the dose response curves were constructed from the mean d output of the last two 15 min periods at each dose level

In series D the degree of inhibition was compared with the disappearance of acid during perfusion of the duodenal bulb in the following way A continuous stimulation with a low dose of gastrin was produced After reaching a secretory plateau the duodenal bulb was perfused with 0 1 HCl or buffer solutions giving three different pH values of the effluent perfusate pH 1 1—1 3 1 7—1 9 and 2 4—2 6 In each experiment the number of milliequivalents of acid delivered to the bulb during the perfusion hour was determined by titration with 0 01 N NaOH using phenolphthalein as indicator During perfusion the effluent solution was collected The volume of the perfusate was measured and by determining its acidity by titration the loss

The

All s a slight pressure ( ) 10 cm H_2O) 100 ml per hour was infused

All perfusions were performed with a calibrated pump The technique of bulbar perfusion has recently been described in detail (Andersson Nilsson and Uvnas 1967)

Statistical evaluation of data

Common methods of analysis of variance were used in the statistical calculation of results (Snedecor 1956)

Results

Series A

In 2 Pavlov and 1 Heidenhain pouch dogs the effect of different pH levels in the duodenal bulb on acid responses to a submaximal dose of gastrin was studied One pH level was studied in each experiment Reduction of the bulbar pH to 2 sup

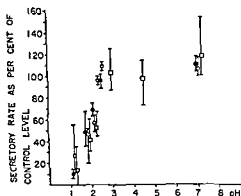


Fig 1

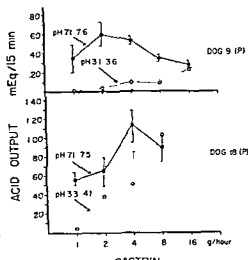


Fig 2

Fig 1 Acid responses to exogenous gastrin in Pavlov (circles) and Heidenhain (squares) pouch dogs at different pHs in the isolated duodenal bulb Each symbol represents the mean of three experiments in one dog Vertical bars show the range pH values refer to the pH of the effluent perfusate

Fig 2 Secretory responses to graded doses of gastrin in two Pavlov pouch dogs during perfusion of the duodenal bulb with buffer solution pH 7 (closed circles) and pH 3 (open circles) Each curve represents the mean of three experiments Vertical bars show the range pH values refer to the pH of the effluent perfusate

pressed the acid response by about 50 per cent Further reduction of the pH in the bulb produced more pronounced inhibition Maximal inhibition occurred when the bulbar pH was 1 1—1 3 (Fig 1)

Series B

The results from this series of experiments are shown in Table I Perfusion of the duodenal bulb with decinormal hydrochloric acid evoked pronounced inhibition of secretory responses to all doses of gastrin The degree of inhibition varied with the dose of gastrin given inhibition being greatest with the lowest dose of gastrin

Series C

The experiments in series B showed that the inhibitory mechanism acted more efficiently the weaker the secretory stimulus This finding suggested that responses to even lower doses of gastrin might be inhibited at pH levels higher than pH 2 0—2 5 Fig 1) Therefore the effect of bulbar perfusion with buffers of pH 3 0 and 7 0 on responses to graded doses of gastrin in 2 Pavlov pouch dogs was studied (Fig 2) The dose of gastrin ranged from 1 to 16 g per hour In both dogs perfusion of the

TABLE I Inhibitory effect of 0 1 N HCl in duodenal bulb on secretory responses to different have been selected to produce secretory responses corresponding to approximately 30.

Dog no	Number of expts	Dose of gastrin g hr	Control hour	
			Range of secretory levels in meq/15 min	Relative S E of mean of four 15-min periods %
6(P)	3	10	0 44—0 51	9
	3	24	0 34—0 58	11
18(P	3	4	0 18—0 42	7
	3	8	0 39—0 67	9
	3	16	0 57—1 29	8
11(H)	3	6	0 17—0.24	6
	3	15	0 37—0 99	6
	3	30	0 66—0 69	7
15 H	3	10	0 16—0 25	6
	3	20	0 18—0 63	14
	3	30	0 36—0 94	10

Acid perfusion of the duodenal bulb was performed during half hour periods 1 and 2 and indicated

duodenal bulb with buffer of pH 3 0 produced pronounced inhibition of responses to 1—4 g gastrin per hour With 8 g gastrin per hour inhibition occurred in one dog and with 16 g per hour no inhibition was found During perfusion the pH of the perfusate rose from pH 3 0 to pH 3 1—4 1

Series D

Table II presents the data from experiments in which the disappearance of hydrogen ions from the duodenal bulb was studied during perfusion with 0 1 N HCl and 2 buffers (pH 1 5 and pH 2 0) giving pHs 1 1—1 3 1 7 —1 9 and 2 4—2 6 respectively in the effluent perfusate No correlation between the disappearance of acid and the degree of secretory inhibition was obtained from these experiments The loss of hydrogen ions was approximately the same from all three solutions but as found previously, the degree of inhibition was dependent on the pH-level

doses of gastrin in Pavlov (P) and Heidenhain (H) pouch dogs The three doses of gastrin 60 and 90—100 per cent of the maximal response to gastrin

Mean and range of secretory rate as a percentage of secretory level during the control hour

Half hour periods following the control hour

1	2	3	4	5
38 (22—52)	10 (6 —15)	39 (23—50)	96 (59—156)	115 (96—133)
82 (67—102)	51 (24—69)	72 (19—105)	133 (84—184)	141 (118—150)
41 (28—66)	15 (10—21)	12 (10—15)	31 (24—38)	102 (88—122)
57 (37—72)	25 (11—50)	24 (7 —46)	56 (13—100)	71 (46—100)
76 (67—92)	41 (36—46)	45 (29—63)	63 (57—70)	61 (48—69)
66 (55—91)	14 (0 —35)	6 (0 —11)	66 (41—80)	108 (96—123)
36 (15—64)	1 (0 —4)	30 (1 —49)	94 (68—131)	105 (100—119)
123 (108—145)	55 (9 —125)	96 (70—141)	77 (54—105)	56 (49—62)
28 (18—34)	0 (0)	18 (0 —34)	33 (7 —52)	97 (74—113)
58 (22—79)	31 (25—39)	59 (33—73)	82 (50—125)	95 (43—161)
75 (64—86)	60 (49—70)	85 (63—102)	77 (64—96)	90 (78—103)

by bold face figures

Discussion

When the intrabulbar pH was varied inhibition of the acid response to a fixed sub maximal dose of gastrin (Series A) did not occur until the pH level within the duodenal bulb was reduced to 2 0—2 5 Further lowering of the bulbar pH reduced the gastric acid output even more If instead the intrabulbar pH was kept constant and the dose of gastrin was varied it was found that inhibition of responses to a small dose of gastrin was greater than inhibition of responses to a large dose These series of experiments indicate that the degree of inhibition is dependent on both the dose of gastrin given and the pH in the bulbar pouch Further support for this con clusion was obtained from experiments in which the inhibitory effect of a higher pH level on responses to graded doses of gastrin ranging from threshold to maximal

doses was studied Thus it could be shown that reduction of the intrabulbar pH to 3 1—4 1 produced significant inhibition at low but not at higher, secretory rates

It is not known how much gastrin is released during a normal digestive cycle However there is evidence that the amounts of gastrin released in response to cephalic stimulation such as sham feeding are rather small. Olbe (1964) showed that the secretory response to sham feeding was greatly reduced by antrectomy in Pavlov pouch dogs The response could be restored to preantrectomy levels by the intravenous infusion of quantities of gastrin which did not stimulate secretion when given alone Gastrin released in response to sham feeding was thus subthreshold for *stimulation of the oxyntic glands and concomitant vagal stimulation was necessary* to demonstrate its stimulatory action During digestion the presence of food in the pyloric antrum also causes release of gastrin It is not known whether the combined action of vagal and local stimulation releases more gastrin than either stimulus alone However it is not necessary to postulate the release of large amounts of gastrin during the digestive phase Firstly gastrin and vagal impulses act synergistically and secondly an optimal degree of potentiation occurs already at threshold amounts of gastrin (Olbe 1964)

Provided that gastrin is released in threshold amounts during digestion one might expect that duodenal inhibition could interfere with secretion even at pH levels higher than 3 Studies on bulbar inhibition of responses to sham feeding have indicated that this may be true Nilsson 1966) Therefore these results question the previous concept that low intraduodenal pH is necessary to activate the duodenal inhibitory mechanism Instead it is possible that the pH levels at which the mechanism starts to operate might be as high as 5

In a recent communication Konturek and Grossman (1965) suggested that inhibition of secretory responses to exogenous gastrin by duodenal acidification was dependent on the quantity of acid delivered to the duodenum and was correlated to the rate of disappearance of hydrogen ions No correlation was found between the pH and the degree of inhibition by these authors They perfused isolated loops of upper duodenum with 0 15 N HCl at rates between 10 and 100 ml per hour With the lowest perfusion rate they were unable to show inhibition despite the fact that the pH of the effluent was 1 8 The rate of disappearance of hydrogen ions was about 0 8 meq per hour With increasing infusion rate the disappearance of acid increased and inhibition occurred

In order to test the hypothesis of Konturek and Grossman (1965) we measured the disappearance of acid during perfusion of the duodenal bulb with acid and two buffer solutions at constant perfusion rates

These experiments showed that the rate of disappearance of hydrogen ions was almost identical in the three solutions On the other hand the pH levels were different and the percentage inhibition of secretion was correlated to the pH as shown in Table II It should be noted that Konturek and Grossman (1965) did not perfuse their duodenal pouches against any pressure This may have resulted in incomplete perfusion of the isolated loops of duodenum during low rates of perfusion Conse-

TABLE II Inhibition of gastric acid secretion and disappearance of acid from the duodenal bulb during one hour of perfusion with solutions of different pH in a Pavlov pouch dog

Instilled solution	Exp no	Secretory rate as a percentage of secretory level during the control hour	pH of effluent perfusate	meq of H^+ delivered	meq of H^+ recovered	loss of H^+ meq
0 1 N HCl	1	13	1 1—1 3	10 19	5 86	4 33
	2	14	1 1—1 3	9 89	6 68	3 21
	3	47	1 1—1 3	8 83	5 62	3 21
	Mean	24	—	9 64	6.05	3 58
Buffer pH 1 5	1	68	1 7—1 9	10 49	7 31	3 18
	2	73	1 7—1 9	10 20	6 87	3 33
	3	79	1 7—1 9	10 40	7 18	3 22
	Mean	73	—	10 36	7 12	3 24
Buffer pH 2 0	1	95	2 4—2 6	10 28	6 69	3 59
	2	104	2 4—2 6	9 89	6 70	3 19
	3	102	2 4—2 6	9 70	6 53	3 17
	Mean	100	—	9 96	6 64	3 32

In all experiments the volumes recovered after perfusion were 3—4 per cent larger than the volume delivered

quently, at higher perfusion rates a larger area of mucosal surface was reached by the acid, giving more efficient acidification of the bulb which resulted in secretory inhibition The importance of controlled perfusion pressure has been emphasized in studies on antral inhibition Andersson and Olbe (1964) found that in some dogs inhibition of vagal release of gastrin by antral acidification could be demonstrated only when the acid had to leave the antral pouch against a slight pressure (10—20 cm H_2O) Similar observations have been made in studies on the stimulatory potency of some chemical agents perfused through the antrum (Elwin, in press)

In our opinion there are no valid arguments against the concept that the duodenal inhibitory mechanism is regulated by the degree of acidity within the duodenal lumen

This investigation was supported by research grants from Statens Medicinska Forskningsråd B 67 14X 88-03, Karolinska Institutets lärarkollegium Svenska Sällskapet för Medicinsk Forskning and Torsten and Ragnar Söderbergs stiftelse

References

ANDERSSON S Inhibitory effects of hydrochloric acid in duodenum on gastrin stimulated gastric secretion in Heidenhain pouch dogs *Acta physiol scand* 1960 *50* 105—11?

ANDERSSON S and B UVNÄS Inhibition of postprandial gastric secretion in Pavlov pouches by instillation of hydrochloric acid into the duodenal bulb *Gastroenterology* 1961 *41* 486—490

ANDERSSON S and L OLBE Inhibition of gastric acid responses to sham feeding in Pavlov pouch dogs by acidification of the antrum *Acta physiol scand* 1964 *61* 55—64

tiated by glass surfaces (Ratnoff and Crum 1966) Activated Hageman factor is then probably releasing kinins via activation of kallikrein in the plasma (Margolis 1958 1960)

Jacobsen's studies on kininogens included plasmas from dog rat and guinea pig but not from cat and hamster Since these latter ones are also common laboratory animals it seemed to be of importance to know more about the different factors involved in formation and inactivation of kinins in their plasmas An analysis of these factors has been carried out in the present study, where some further examination of guinea-pig plasma has also been included It was particularly intended to study the specificity of the two kininogens in the various plasmas towards different kinin forming agents and procedures The possibilities of influencing the concentration of the kininogens *in vivo* by i v injection of ellagic acid were also examined

Methods

Plasma kinin activity was tested on the isolated rat uterus preparation Virgin rats weighing 150—200 g were given 30 µg stilboestrol intraperitoneally 20 hr before being sacrified In order to reduce the spontaneous activity of the muscle and also to make it insensitive to kallikreins the isolated uterus was stored for 24 hr at 4° C in de Jalon solution before being used A 2 cm piece of one uterine horn was used for each test series The load on the muscle was 1 1—1 3 g when kinin formation was studied and 1 8—2 4 g when kininase activity was estimated The tests were carried out with the uterus preparation suspended in a 5 ml organ bath containing aerated de Jalon solution at 29° C The contact time was 1 min and the intervals between tests were 4 or 5 min The preparation was sensitive to the addition of as little as 1 ng of bradykinin in tests where kinin formation was studied

Kininase activity was estimated by incubating at 37° C 500 ng of synthetic bradykinin (BRS 640 Sandoz Basle Switzerland) dissolved in 0 1 ml of saline with 0 9 ml of the fluid to be tested Aliquots of 0 1 ml of the mixture were taken out for testing on the rat uterus every 5 min The first test was made 1 min after the start of the incubation The amount of bradykinin in the test samples was estimated by comparing the contraction of the rat uterus preparation caused by the addition of the sample and that caused by known amounts of synthetic bradykinin *One unit of kininase* was defined as the amount of enzyme which destroyed 75 % of 500 ng of bradykinin in 11 min under standardized conditions (Rugstad 1966) If the plasma to be tested for kininase activity contained more than 1 unit of kininase per 0 9 ml that is if 0 9 ml of it needed less than 11 min to break down 75 % of 500 ng of bradykinin then the sample was diluted with 0 02 M sodium phosphate buffer of pH 7 5 The most diluted solution where 0 9 ml still inactivated at least 75 % of the bradykinin in 11 min was said to contain 1 unit of kininase in these 0 9 ml On the basis of this the amount of kininase in the original undiluted test sample was calculated The precision of the method was tested by estimating kininase activity as described above in 7 samples of each of 2 different plasmas The results of these tests are given in Table I

Plasmas from man cat hamster and guinea pig were obtained from citrated blood one part of 3 1 % sodium citrate dihydrate solution to nine parts of blood) taken with and prepared in siliconated equipment All blood samples were centrifuged at 1500 × g for 30 min

Plasma kallikrein preparation Pseudoglobulin preparations were used as a source of plasma kallikrein Citrated human plasma was precipitated with ammonium sulphate between 33 and 46 % saturation as described by Lewis (1958 The precipitate was dissolved in distilled water and dialysed against running tap water for 20 hr The final volume of the preparation was about one third of the original plasma volume The pseudoglobulin samples thus prepared contained less than one unit of kininase activity and this was completely inhibited by disodium edetate dihydrate or by phenanthroline The plasma kallikrein preparation which could be shown to contain no substrate for plasma kinin forming enzymes was stored at −20 C

Glandular kallikrein Saliva from guinea pig hamster and cat was used as a source of glandular kallikrein In these experiments plasma from the different species was incubated

anesthesia and after stimulation

The saliva was centrifuged and

y Amundsen and Nustad (1964)

TABLE I Determination of kininase activity precision of the method

Sample	Units of kininase activity in test plasma 1	Units of kininase activity in test plasma 2
I	13	210
II	14	218
III	17	230
IV	13	220
V	17	215
VI	15	215
VII	16	216
Mean	*15*	*216*
Standard deviation	*2 3* (15 3 %)	*7 9* (3 2 %)

Kininase activity was estimated as described in the text in 7 plasma samples from each of two different animals Test plasma 1 and 2 is from cat and guinea pig respectively

... in forming enzymes Before being used ... dihydrate (A.G Buchs, Switzerland) ... iroline, Sigma Chemical Company, St ... idard solutions were dissolved in 0 05

Determination of kininogens in plasma The presence and specificity of substrate 1 and 2 ... of 0 8 ml from cat, ... (15 min—48 hr) ... b) 0 4 ml of the ... exposed to massive glass contact for various time intervals, and any kinin formation observed When no more kinin activity or only traces of such activity could be detected after any of these procedures, 0 2 ml of the mixture was incubated for 5—10 min with 0 1 ml of a kininase inhibitor and the final incubate tested for ability to form kinin on addition of in turn, 0 1 ml of the kinin-forming agents previously described or by exposing the incubate to massive glass contact.

In a new set of tests on plasma from each species the total amount of kinins which could be developed on incubation with a) human plasma kallikrein preparation, with b) the animal s ...

amounts of substrates 1 and 2 were then calculated taking into account the reaction pattern of the various plasmas towards the two types of kallikreins Development of kinin with the plasma kallikrein preparation and on contact with glass was taken as evidence for the presence in the plasma of substrate 1 The development of a different amount of kinin on addition to the plasma of the animal s own saliva was taken as evidence for the presence of a substrate of type 2 In preliminary experiments it was shown that maximal kinin values in the various reaction mixtures occurred after 5 to 20 min of incubation and in the subsequent experiments aliquots of 0 1 ml were therefore tested for kinin formation within this interval of time and the highest kinin value obtained was used

Each substrate was determined quantitatively in terms of kinin that was formed per ml of plasma synthetic bradykinin being used as reference The evaluation of the level of the two substrates was carried out separately by socalled bracketing the dose ratio being 2 3 (Gautvik and Rugstad 1967)

Recording of respiration and of blood pressure during intravenous injection of ellagic acid Hamsters (85—120 g and guinea pigs 250—400 g) were anesthetized by i p injections of

40—50 mg/kg b.w. of pentobarbitone (Nembutal Abbot Laboratories London). Anesthesia was maintained by further injections of 5×10 mg/kg b.w. as needed. The trachea was cannulated and injections were made through a catheter inserted into the external jugular vein. The blood pressure was recorded via a cannula in the carotid artery with a pressure transducer (P 23 De, Statham) connected to a Sanborn model 320 Dual channel DC amplifier—recorder (Sanborn Company, Massachusetts USA) equipped with a Sanborn preamplifier. The frequency of respiration was followed by insertion of a constantan—copper thermocouple in the tracheal cannula. The thermocouple was connected to a Beckman preamplifier, type 461, which was again connected to a Beckman type RS Dynograph.

Glass activation of plasma. This was carried out in plasma or in mixtures of plasma and a kininase inhibitor by agitating equal volumes of such samples and glass beads of diameter 0.1—0.2 mm for various time intervals (1 min to 24 hr).

Ellagic acid (4.4', 5.5', 6.6' hexahydroxydiphenic acid 2.6, 2'.6' dilactone (Fluka AG Buchs Switzerland) was used in a standard solution 2×10^{-4} M in a 0.15 M Tris-HCL buffer of 7.35.

Dialysis was carried out with a Visking dialysis tubing 18/32 (Visking Dept., Union Carbide International Co. New York, USA).

Siliconizing of glassware and needles was carried out with Siliclad (Clay Adams Inc. New York USA). A 1 % solution was used for glass and 5 % solution for metal followed by drying over night at 100° C.

Results

Kininase activity in plasmas from cat, hamster and guinea pig

The kininase activity in plasmas from these animals was estimated (see Methods) and found to vary considerably as shown in Table II. The efficiency of disodium edetate and phenanthroline as plasma kininase inhibitors was also determined. In hamster and guinea pig the plasma kininase activity was inhibited most efficiently by phenanthroline while both types of inhibitors were highly efficient towards cat plasma kininase. When kinin formation was studied in hamster and guinea pig plasmas, phenanthroline was therefore used as a kininase inhibitor. In cat plasma disodium edetate was used.

TABLE II. Plasma kininase activity

Type of animal	Number of animals	Kininase activity in plasma (range)*		
		without inhibitor	with inhibitor present	
			disodium edetate	phenanthroline
Cat	8	1—20	less than 1	less than 1
Hamster	10	10—[illegible]0	2—5	1—2
Guinea pig	10	160—220	4—8	1—4

*Values are given as units of kininase activity
for definition of a unit, see Methods

Kininase activity in plasmas from cat, hamster and guinea-pig was estimated as described in Methods

TABLE III *Reaction pattern of substrates 1 and 2 towards various kinin-forming agents*

Source of plasma		Incubation with			On glass activation
		human plasma kallikrein	species specific saliva	ellagic acid	
Cat	substrate 1	+	0 (+)*	+	+
	substrate 2	0	+	0	0
Hamster	substrate 1	+	+	+	+
	substrate 2	0	+	0	0
Guinea pig	substrate 1	+	0	+	+
	substrate 2	+	+	0	0

*In 2 out of 5 animals the concentration of substrate 1 was found to be somewhat reduced after 24 hrs of incubation

Incubations of the kinin forming agents with pre treated plasma from various animals, in the presence of a kininase inhibitor, were carried out as described in Methods, and the kinin activity *was tested on the isolated rat uterus preparation*

\+ = marked formation of kinin activity which took place in the course of a few minutes
(+) = a small formation of kinin activity which took place in the course of hours
0 = no kinin activity was detected even on prolonged incubation

The presence and specificity of substrates 1 and 2 towards various kinin-forming agents

The presence of substrate 1 and 2 in plasma from cat, hamster and guinea-pig was confirmed and their abilities to form kinins with human plasma kallikrein, with the animals' own salivary kallikrein on addition of ellagic acid and on exposure to massive glass contact were determined as described in Methods In Table III are shown the results obtained from the various types of plasmas In cat and hamster human *plasma kallikrein releases kinins from substrate 1 only whereas in guinea-pig* it attacks both substrates, as previously shown by Jacobsen (1966b) In cat and guinea-pig saliva from the actual species acts upon substrate 2 only On prolonged incubation of cat plasma and such glandular kallikrein (24 hrs) some reduction in the substrate 1 level was occasionally found (in 2 plasmas out of 5 tested) Hamster saliva induced kinin formation from both substrates in plasma from this animal In *the various plasmas tested ellagic acid and glass activation released kinins* from substrate 1 only Addition of ellagic acid or glass activation did deplete cat and hamster plasmas for substrate 1 whereas the substrate 1 concentration was only reduced in plasma from guinea pig by such procedures

The amounts of substrate 1 and 2 in plasmas from cat, hamster and guinea-pig

In cat plasmas the concentrations of substrate 1 and 2 were determined as described in Methods simply by measuring kinin formation on incubation with human plasma

TABLE IV The contents of substrate 1 and 2 in plasmas from cat, hamster and guinea pig

	Number of plasmas tested	Substrate 1		Substrate 2	
		Range	Mean	Range	Mean
Cat	12	150—450	280	500—2000	1200
Hamster	10	400—900	550	600—1700	1100
Guinea pig	9	300—700	400	100— 250	150

The concentration of substrate 1 and 2 are given as amounts of kinin (ng/ml) that could be developed with an excess of kinin forming enzymes

The amounts of kinin formed are again expressed in terms of comparable amounts of synthetic bradykinin and using a bracketing assay as described in Methods The values for substrate 1 and 2 are obtained as described in Results In the table the range and mean values are given for each plasma

kallikrein on the one hand and with the animals own salivary kallikrein on the other In hamster plasmas the substrate 2 levels were calculated as the difference in amounts of kinin released pr ml plasma by glandular kallikrein and by human plasma kallikrein respectively In guinea pig plasma the level of substrate 1 was calculated as the difference in amounts of kinins formed by human plasma kallikrein on the one hand and by the animal s own saliva on the other Table IV gives the range and the mean values for the kininogen levels in the various plasmas In cats the amount of substrate 1 corresponds to about 1/4—1/5 and in hamsters to about 1/3 of the total amount of kininogens In guinea pig plasmas however the substrate 1 level equals about 2/3 of the total amounts of kininogens present

Kinin formation by ellagic acid

When ellagic acid is added to a mixture of rat plasma and disodium edetate marked kinin like activity developes Evidence for the kinin nature of the substance(s) formed has (have) been presented by Gautvik and Rugstad (1967) When 0 1 ml of a standard solution of ellagic acid was incubated with 0 2 ml of plasma from cat hamster or guinea pig in the presence of 0 1 ml of a kininase inhibitor kinin activity could similarly be detected on addition of 0 1 ml of this mixture to the organ bath In cat plasma kinin activity corresponding to 150—400 ng pr ml plasma (average 300 ng ml) was observed and the maximal amounts developed within 20—25 min of incubation In plasmas from guinea pig and hamster ellagic acid induced kinin formation corresponding to 200—600 ng pr ml with average values of 300 and 500 ng/ml respectively The maximal kinin values occurred in both these plasmas within 10 to 15 min of incubation

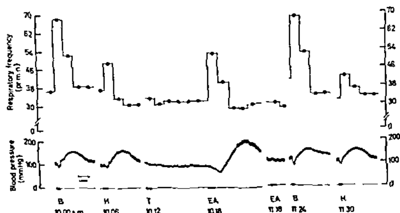


Fig 1 The effect of repeated intravenous injections of ellagic acid on respiration and blood pressure in an anesthetized hamster

Intravenous injections of synthetic bradykinin, 10 μg/kg (B), of histamine, 20 μg/kg (H), of 0 3 ml Tris HCl buffer (T) or of 0 3 ml 2×10^{-4} M ellagic acid (EA) were carried out at signals The effects of the first and of the last of 4 successive injections of ellagic acid are shown and compared to those of synthetic bradykinin and histamine The intervals between the ellagic acid injections were 15 min

Effects of intravenous injection of ellagic acid on blood pressure, respiration and kininogen levels in hamster and guinea-pig

A standard solution of ellagic acid was injected into the jugular vein of 7 hamsters and 7 guinea-pigs Injections of 0 3 ml and 0 5 ml of a 2×10^{-4} M solution were given to the two types of animals, respectively, usually at intervals of 8—15 min In Fig 1 is shown the effects on blood pressure and respiration during a typical experiment with a hamster From 5—20 sec after the first injection the mean blood pressure was reduced by 20—40 mm of Hg. The hypotension usually lasted for 1—4 min, after which a longer lasting hypertensive period followed, before the pressure returned to its initial value After the 3rd or 4th injection of ellagic acid these effects on blood pressure could no longer be observed Control injections of 0 3 ml of Tris-HCl buffer, the medium in which ellagic acid was dissolved had no effect on blood pressure or on respiration The effects of intravenous injections of histamine or of synthetic bradykinin were, however, apparently unchanged after several injections of ellagic acid Marked increases in the frequency of respiration could be observed following the injections of ellagic acid of histamine or of synthetic bradykinin

In Fig 2 is shown the same type of experiment performed on a guinea-pig The hypotensive effect of an intravenous injection of 0 5 ml of ellagic acid subsided on repeated injections, and a 5th or 6th injection had no such effect Control injections of the Tris-HCl solution had no effect on the blood pressure or respiration The effects of intravenous injections of histamine or of synthetic bradykinin were about the same before and after the injections of ellagic acid Also in guinea-pigs an increase in the frequency of respiration was found during the hypotensive periods induced by ellagic acid

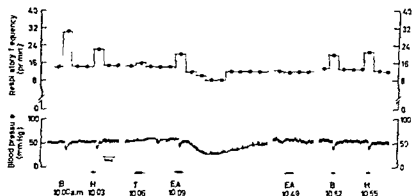


Fig 2 The effect of repeated injections of ellagic acid on respiration and blood pressure of an anesthetized guinea pig

Intravenous injections of synthetic bradykinin 6 μg/kg (B) of histamine 10 μg/kg (H) of 0 5 ml Tris-HCl buffer or of 0 5 ml ellagic acid 2×10^{-4} M (EA) were carried out at signals. The effects of the first and last of 5 successive injections of ellagic acid are shown and compared to those of synthetic bradykinin and histamine The intervals between ellagic acid injections were 8 min

In arterial plasma samples from guinea pig taken after repeated injections of ellagic acid the addition of ellagic acid or performance of the glass activation did usually not cause any kinin formation Addition of human plasma kallikrein or of species specific saliva did however still induce release of kinin there thus being some ...nts of the two substrates left

...e levels of substrate 1 and 2 in plasma of hamsters before and after injections of ellagic acid

In 13 animals one 0 8 ml arterial blood sample was taken before the intravenous injection of 4 doses of 0 3 ml of 2×10^{-4} M ellagic acid given at 15 min intervals Additional similar samples were taken from each hamster after these injections The concentrations of substrate 1 and 2 in the samples were determined separately as described previously The plasma amount of each substrate before any injection of ellagic acid was said to be a 100 % level and all later values were related to this level In all hamsters but one a very reduced amount of substrate 1 could be detected 15 min after the last of the 4 injections In Fig 3 are shown the mean plasma levels of substrate 1 and 2 at various time intervals after the last ellagic acid injection One hr after the last injection the level of substrate 1 was in all 13 hamsters reduced to between 0 and 20 % (mean 13 %) of the initial level After 6 hr the mean concentration of substrate 1 had reached 31 % and after 24 hr the mean level of this particular substrate had reached 83 % of the initial value The concentration of substrate 2 was followed in 4 animals and could not be found to be significantly altered by the injections

In two sham-operated animals to which intravenous injections of a Tris HCl solution were given instead of ellagic acid there were no changes in the substrate concentration

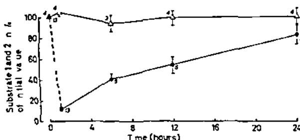


Fig 3 The levels of the substrate for plasma kallikrein (substrate 1) and the substrate for glandular kallikrein (substrate 2) in plasma of hamsters before and after intravenous injection of ellagic acid Results from 13 hamsters are given in this figure The point of the last of four injections of 0 3 ml of a buffered 2×10^{-4} M solution of ellagic acid is given at zero time The mean values of substrate 1 (closed circles) and of substrate 2 (triangles) levels in arterial blood samples and the standard deviation of these means are given The substrate levels are expressed as percent of the initial pre-injection blood value The substrate amounts have again been evaluated as the amount of kinin formed per ml of plasma, synthetic bradykinin being used as reference (see Methods) The number of animals from which blood samples were taken and analyzed at each different point is given in italics

Discussion

The amounts of substrate 1 and 2 found in the various experiments must be considered as minimum values since disodium edetate and phenanthroline did not inhibit completely the kininase activity of the plasmas The values given for the two substrates have not been corrected for the small loss of kinin activity which thus probably occurred during incubation

In all plasmas so far examined ellagic acid induced kinin formation The addition of this agent seems to be a convenient way of releasing kinins *in vitro* as well as *in vivo* The agent seems to operate by activating the plasma kallikrein present, and thus separately influencing the concentrations of substrate 1

The effects in hamster and guinea pig of ellagic acid injected intravenously on blood pressure and respiration are also consistent with the view that the agent causes release of kinins (Gautvik and Rugstad 1967) On repeated injections these effects subside probably as a result of the amount of kininogen available (substrate 1) being markedly reduced

In hamsters the initial drop in blood pressure caused by intravenous injections of bradykinin histamine and ellagic acid was followed by a marked hypertensive period Lang and Pearson (1968) reported that bradykinin and kallidin produced biphasic blood pressure responses when injected intravenously or into the carotid artery in anesthetized cats They concluded that the pressor responses seen were mediated by sympathetic mechanisms involving a central nervous component

When ellagic acid was added to plasma of cat or hamster *in vitro*, no substrate 1 could be detected 15 min later When portions of guinea pig plasma were preincubated with ellagic acid for various time intervals (15 min to 25 hr) addition of human plasma kallikrein or the species specific glandular kallikrein would however, still induce kinin formation Similarly massive glass activation could not deplete guinea pig plasma of its substrate 1 Kinin formation caused by ellagic acid and glass activation involves intermediate factors The findings mentioned above indicate that

some intermediate factor(s) needed for the action of ellagic acid and glass is (are) exhausted early in the kinin yielding process. Jacobsen (1966b) has previously reported that guinea pig plasma was depleted of both substrates on incubation with ellagic acid. He did apparently use a rat uterus preparation with lower sensitivity towards added synthetic bradykinin, and this may explain the discrepancy.

The total amounts as well as the relative concentrations of substrate 1 and 2 vary to a considerable extent from one species to another. This may indicate possible differences in physiological or pathophysiological roles of the kinin system in the different kinds of animals.

The intravenous administration of ellagic acid to hamsters reduced the concentration of substrate 1, leaving the amount of substrate 2 unchanged. These results make it unlikely that any complete interconversion between the two substrates takes place in that animal.

The author wants to thank Solveig Strand and cand. mag. Tor Stovne for skilful technical assistance. Financial support from the Norwegian Research Council for Science and the Humanities, from Nansen Foundation and from the Norwegian Council on Cardiovascular Diseases is gratefully acknowledged.

References

AMUNDSEN, E. and K. NUSTAD: Kininase activity of human saliva. *Nature* (Lond.) 1964. *201*. 1226—1227.

FASCIOLO, J. C. and K. HALVORSEN: Specificity of mammalian kallinogen. *Amer. J. Physiol.* 1964. *207*. 901—905.

GAUTVIK, K. M. and H. E. RUGSTAD: Kinin formation and kininogen depletion in rats after intravenous injection of ellagic acid. *Brit. J. Pharmacol.* 1967. *31*. 390—400.

JACOBSEN, S.: Substrates for plasma kinin forming enzymes in human, dog and rabbit plasmas. *Brit. J. Pharmacol.* 1966a. *26*. 403—411.

JACOBSEN, S.: Substrates for plasma kinin-forming enzymes in rat and guinea pig plasmas. *Brit. J. Pharmacol.* 1966b. *28*. 64—72.

LANG, W. J. and L. PEARSON: Studies on the pressor responses produced by bradykinin and kallidin. *Brit. J. Pharmacol.* 1968. *32*. 330—338.

LEWIS, G. P.: Formation of plasma kinins by plasmin. *J. Physiol.* (Lond.) 1958. *140*. 285—300.

MARGOLIS, J.: Activation of plasma by contact with glass: evidence for a common reaction which releases plasma kinin and initiates coagulation. *J. Physiol.* (Lond.) 1958. *144*. 1—22.

MARGOLIS, J.: The mode of action of Hageman factor in the release of plasma kinin. *J. Physiol.* (Lond.) 1960. *151*. 238—252.

RATNOFF, O. D. and J. C. CRUM: Activation of Hageman factor by solutions of ellagic acid. *J. Lab. clin. Med.* 1964. *63*. 359—377.

RUGSTAD, H. E.: Kininase production by some microbes. *Brit. J. Pharmacol.* 1966. *28*. 315—323.

Acta physiol. scand. 1969. 76. 201—212

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Dissociation of Histamine Release and $^{22}Na^{+}$ Uptake in Rat Mast Cells Exposed to Compound 48/80 in vitro

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Received 22 August 1968

Abstract

SLORACH, S. A. and B. UVNÄS. *Dissociation of histamine release and $^{22}Na^{+}$ uptake in rat mast cells exposed to compound 48/80 in vitro.* Acta physiol. scand. 1969. 76. 201—212.

[illegible]

sodium for histamine does not play a significant role in histamine release induced by compound 48/80. The large increase in $^{22}Na^{+}$ uptake by mast cells exposed to compound 48/80 can be prevented by pretreating the cells with N-ethylmaleimide or ninhydrin, which also inhibit the histamine release. We found evidence for the existence of a "sodium pump" mechanism which could be inhibited by ouabain. The histamine liberator d-tubocurarine also produced an increase in the net $^{22}Na^{+}$ uptake by mast cells but another releaser, n-decylamine, which produces histamine release by lysis of the cell membrane, did not.

In rat peritoneal mast cells histamine is stored in discrete intracellular granules together with protein and heparin. Åborg and Uvnäs (1968) have presented evidence that the histamine in such granules is electrostatically linked to the protein part of the protein-heparin complex via carboxyl groups and it can be released by suspending the granules in cation-containing media (Åborg, Novotný and Uvnäs 1967). It seems probable that the histamine present in mast cell granules *in situ* is stored in a similar manner to that in isolated granules; this is a prerequisite for the 'ion-exchange' mechanisms of histamine release induced by compound 48/80 proposed by various authors (*vide infra*).

Thon and Uvnäs (1967) proposed that the response of rat mast cells to compound 48/80 took place in two steps. The degranulation step, the primary event, is the result of the triggering of an 'active' energy-requiring transport of histamine-con-

taining granules to the outside of the cell. The release of histamine then occurs *extracellularly* as a cation exchange between the amine in the discharged granules and cations in the extracellular medium. Accordingly, if the extracellular medium is cation free (e.g. isosmotic sucrose) histamine-containing granules can be recovered by differential centrifugation.

Another hypothesis is that sodium ions pass into the mast cells and release histamine from the granules by *intracellular* cation-exchange; this theory lacks experimental support. If it were true the release of histamine should take place simultaneously with or after the uptake of sodium ions from the extracellular medium and *the amount of sodium (atoms) taken up should be equivalent to or greater than* the amount of histamine (molecules) released.

In preliminary experiments we found that the exposure of mast cells to compound 48/80 led to an uptake of ^{22}Na and a release of histamine. We wished to find out if this uptake of sodium ions could bring about the histamine release by an *intracellular* ion-exchange. In order to do this we have investigated whether or not there was any quantitative or temporal correlation between sodium uptake, degranulation and histamine release in isolated rat mast cells exposed to compound 48/80. We have also investigated the effects of incubation temperature and time, compound 48/80 concentration, enzyme inhibitors (N-ethylmaleimide, ninhydrin and ouabain) and repeated exposures to compound 48/80 on the ^{22}Na uptake by rat mast cells *in vitro*. In addition, we compared the effects of compound 48/80 and two other histamine releasing agents, d-tubocurarine and n-decylamine, on ^{22}Na uptake by mast cells.

Methods and materials

Isolation of mast cells

Mast cells were obtained from the peritoneal and pleural cavities of male Sprague-Dawley rats (3[illegible] g) and separated from other cells by density gradient centrifugation in Ficoll according to the method of Thon and Uvnäs (19[illegible]). Cell counts were performed in a Bürker chamber after staining with toluidine blue.

Incubation and washing procedure

a. general method

Mast cells ([illegible]) were incubated in a salt solution (NaCl 154 mM, KCl [illegible] mM, $CaCl_2$ 0.9 mM) containing 10 % v/v Sörensen phosphate buffer (Na_2HPO_4–KH_2PO_4 6.7 × 10^{-2} M, pH 7.0) and 1 mg human serum albumin per ml. The incubations were carried out in a volume of 1 ml in plastic tubes (id. 9 mm, length 5 cm) with tightly fitting plastic stoppers. $^{22}NaCl$ solution (ca 1 μc/ml) was added to the cell suspension to give a final level of radioactivity of approximately 1 μc/ml. This level of radioactivity gave [illegible] × 10^{6} cpm per ml when measured as described later. Unless otherwise stated, all other reagents were added to the cell suspensions immediately after adding the $^{22}NaCl$ solution and immediately prior to incubating the cells at 37 °C for [illegible] min.

After incubation the tubes were centrifuged at 3[illegible] × g for 1[illegible] min. The supernatants were poured off as completely as possible and the residual fluid was removed using small rolls of tissue paper. The cells were then washed by resuspending them in 1 ml of buffered salt solution (wide supra), centrifuging and removing the supernatants as described above. When these washes were carried out at 0 °C, [illegible] °C or 37 °C it was found that at the two latter temperatures the net ^{22}Na uptake was [illegible] less than when the cells were washed at 0 °C. For this reason the tubes were kept at 0 °C during all the procedures carried out after the incubation. After the three washes the cells were suspended in 1 ml of buffered salt solution and heated at 9[illegible] °C for 10 min.

In a typical experiment the levels of radioactivity (per ml, above background) were as follows incubation supernatant 7×10^6 cpm first wash $20—35 \times 10^3$ cpm second wash 150—400 cpm third wash 60—200 cpm and heated washed cell suspension 200—3 000 cpm From

that

gible showing that the quantity of $^{22}Na^+$ retained by the tubes after three washes was insignificant

(b) method for time course studies

(1967) in their study of the time course of histamine release induced by compound 48 80 After centrifuging the cells were washed twice with 10 ml quantities of buffered salt solution using a procedure similar to that in the general method The washed cells were then heated in 1 ml of buffered salt solution at 70° C for 10 min

Liquid scintillation counting

The radioactivity in 0 5 ml samples of the washes and the heated cell suspensions and in 0 05 ml samples of the original supernatants was determined after dissolution in 15 ml of scintillator solution using a Tri Carb liquid scintillation spectrometer Packard Instrument Co Inc La Grange Ill, USA The scintillator solution consisted of a solution of 0 4 % 2 5 diphenyloxazole and 0 01 % 1 4 bis 2 (4 methyl 5 phenyloxazolyl) benzene in a mixture of equal volumes of toluene and ethylene glycol monoethyl ether In the results a correction has been made for background radioactivity (~45 cpm) but not for quenching or counting efficiency (>70 %)

Histamine assay

Histamine was measured fluorometrically according to the method of Shore, Burkhalter and Cohn (1959) but the purification steps were omitted and o-phthalaldehyde was added directly to the samples after alkalinization (Fredholm and Haegermark 1967)

Materials

Ficoll was obtained from AB Pharmacia,
from preservatives) from AB Kabi Strangi
by Dr B Hogberg AB Leo Halsingborg
9 mc/mg Na was obtained from the Rac
octahydrate was obtained from the Sigma

All other materials were obtained from normal commercial sources

Results

In our experimental procedures we have measured the amount of ^{22}Na taken up by the mast cells during the incubation period and retained by them after washing We will refer to this as net ^{22}Na uptake throughout this paper

Factors affecting net ^{22}Na uptake by mast cells treated with compound 48/80

Incubation temperature

In this experiment the cell suspensions were preincubated for 10 min at the required temperatures before adding compound 48 80 Table I shows the effect of different incubation temperatures on the net uptake of Na by normal mast cells and mast

TABLE I Effect of incubation temperature on net $^{22}Na^+$ uptake by normal rat mast cells and mast cells treated with compound 48/80 (1 μg/ml) Means o duplicates and range

Incubation temperature	*Net ^{22}Na Uptake* Normal cells	*(cpm/10⁶ cells)* Cells + 48/80
0° C	316 (274—358)	444 (413—476)
37° C	377 (365—389)	2003 (1917—2090)
46° C	529 (497—562)	493 (490—497)

cells exposed to compound 48/80 (1 μg/ml) Uvnas and Thon (1961) reported that histamine release induced by compound 48/80 (0 5 μg/ml) from such cells showed a similar temperature dependence It did not take place at 0° C or above 44° C and was optimal at 37° C

Concentration of compound 48/80

The effect of increasing concentrations of compound 48/80 on the net uptake of ^{22}Na by and the release of histamine from mast cells is shown in Fig 1 The two dose-response curves show obvious similarities

Incubation time

When the procedure for time course studies (*vide Methods*) was used the maximal net ^{22}Na uptake was found after about 10 min incubation (Fig 2) Furthermore, in studies with very short incubation periods it was shown that there was a latent period of about 5 sec before uptake began (Fig 3)

In another series of experiments $^{22}NaCl$ was added to the incubation mixture before simultaneously with or after the addition of compound 48/80 The incubations were stopped 20 min after the time that both $^{22}NaCl$ and compound 48/80 had been added and the general washing procedure was used It can be seen (Fig 4) that the addition of $^{22}NaCl$ before or simultaneously with compound 48/80 results in ap-

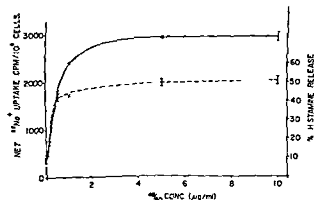


Fig 1 The effect of compound 48/80 concentration on the net uptake of $^{22}Na^+$ by (full line) and the release of histamine from (broken line) rat mast cells Each point represents the mean of duplicates vertical lines indicate range

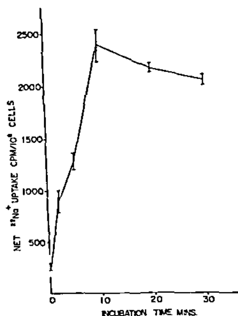


Fig 2

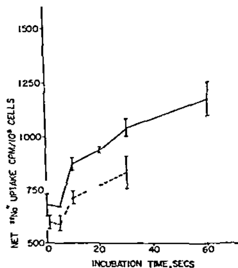


Fig 3

Fig 2 The effect of incubation time on the net uptake of $^{22}Na^+$ by mast cells exposed to compound 48/80 (1 μg/ml) Method b Each point represents the mean of duplicates, vertical lines indicate range

Fig 3 Demonstration of the lag phase in the uptake of $^{22}Na^+$ by mast cells treated with compound 48 80 (1 μg ml) Method b Each point represents the mean of duplicates, vertical lines indicate range Full lines Expt I, broken lines Expt II

N B Histamine release begins after ca 5 sec and is complete after ca 30 sec

proximately the same net uptake Addition of ^{22}NaCl at times after the addition of compound 48/80 results in a decreased ^{22}Na uptake It can be seen that the rate of sodium uptake is most rapid initially and decreases progressively with time

Repeated exposure to compound 48/80

a) Fig 5 shows the net ^{22}Na uptake by normal mast cells and mast cells exposed once or twice to compound 48/80 (1 μg/ml each exposure) with different numbers of intervening washes The ^{22}Na was added before the *last* exposure to compound 48/80 and thus in the case of double exposures to compound 48/80 it is the uptake in response to the *second* exposure that is recorded It can be seen that although the net uptake is less after a second exposure to compound 48/80 (compared to that after a single exposure) it is always substantial This effect on the ^{22}Na uptake contrasts with the degranulation and histamine release response on repeated exposure of mast cells to compound 48/80 Thon and Uvnäs (1967 showed that in the absence of an intervening wash the mast cells did not respond with degranulation or histamine release on the second exposure to compound 48/80 After washing the cells successively regained their a!

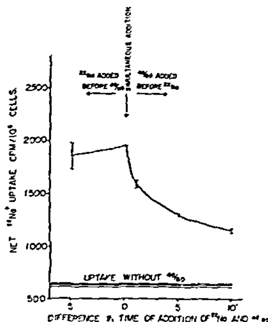


Fig 4

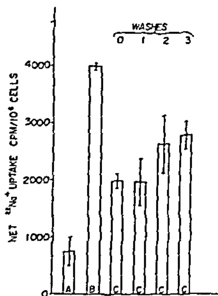


Fig 5

Fig 4 Net $^{22}Na^+$ uptake by mast cells exposed to compound 48/80 (1 µg/ml) The $^{22}NaCl$ was added at the times indicated. Incubations for 20 min after both $^{22}NaCl$ and 48/80 were present in the incubation mixture Method a. Each point represents the mean of duplicates, vertical lines indicate range

Fig 5 Net $^{22}Na^+$ uptake by normal mast cells (A) and mast cells exposed once (B) or twice (C) to compound 48/80 (1 µg/ml) with different numbers of intervening washes $^{22}NaCl$ added before last exposure to compound 48/80 Each bar represents the mean of four experiments, vertical lines indicate S D

b) Table II shows the net $^{22}Na^+$ uptake by, and histamine release from untreated mast cells and mast cells exposed once or twice to compound 48/80 (1 µg/ml) without an intervening wash In this experiment the $^{22}NaCl$ was added before the *first* exposure to compound 48/80 In the case of the double exposure to compound 48/80 both the histamine release and the net $^{22}Na^+$ uptake are the total values for both the exposures The histamine release in response to the second exposure to compound 48/80 (1 µg/ml) is negligible whereas the first exposure to the same concentration releases 43 % of the histamine from the cells It should be noted that the net uptake of $^{22}Na^+$ is slightly less in the cells exposed twice to compound 48/80 than in the cells treated only once, the significance of this will be dealt with in the Discussion.

Enzyme inhibitors

N-Ethylmaleimide (NEM) (10^{-4} M) The influence of this -SH reacting enzyme inhibitor on the net uptake of $^{22}Na^+$ by normal mast cells and mast cells treated with compound 48/80 (1 µg/ml) is shown in Table III This concentration of NEM is sufficient to prevent histamine release from mast cells induced by compound 48/80 Mast cells treated with NEM alone show an increase in net ^{22}Na uptake

TABLE II Net ^{22}Na uptake by and histamine release from normal mast cells and mast cells exposed once or twice to compound 48/80 (1 μg/ml) without an intervening wash ^{22}NaCl added before the first exposure to the releaser Means of duplicates and range

Cell treatment	*^{22}Na uptake**	*Histamine release***
Normal cells	802 (755—850)	9 % (9 9)
Cells + 48/80-one exposure	5959 (5918—6000)	43 % (41—45)
Cells + 48/80-two exposures	4930 (4503—5367)	44 % (42—46)

*Net ^{22}Na uptake cpm per 10^6 cells
**As a percentage of the total histamine content of the cells

TABLE III Effect of N-ethylmaleimide (NEM) (10^{-4} M) on the net ^{22}Na$^+$ uptake by mast cells and mast cells exposed to compound 48/80 (1 μg/ml) Means of duplicates and range

Cell Treatment	*Net ^{22}Na uptake* (cpm/10^6 cells)
Untreated cells	667 (642—697)
Cells + NEM	1221 (1139—1303)
*Cells + NEM + 48/80**	*1388 (1378—1398)*
Cells + 48/80	4410 (4194—4627)
Cells + 48/80 + NEM**	4860 (4303—5418)

*Treated 5 min 20° C with NEM before adding 48/80
**Treated 5 min 37° C with 48/80 before adding NEM

compared with untreated cells Cells pretreated with NEM and then exposed to compound 48/80 show no increase in uptake above that of cells treated with NEM alone Addition of NEM 5 min after compound 48/80 does not decrease the net amount of ^{22}Na taken up compared with that taken up by cells treated with compound 48/80 alone

Ninhydrin

The amino group reagent ninhydrin (10^{-4} M) produced very similar effects on the ^{22}Na uptake by normal mast cells and mast cells treated with compound 48/80 (1 μg/ml) (Table IV) to those reported above for NEM This concentration of ninhydrin is sufficient to block histamine release induced by compound 48/80 (Uvnas and Thon 1961)

Ouabain

a) When the uptake of ^{22}Na by normal mast cells and mast cells treated with ouabain (10^{-4} M) was investigated using the general method the net uptake of

TABLE IV Effect of ninhydrin (N) (10^{-4} M) on the net $^{22}Na^+$ uptake by mast cells and mast cells exposed to compound 48/80 (1 µg/ml) *Means of duplicates and range*

Cell treatment	*Net $^{22}Na^+$ Uptake* (cpm/10^6 cells)
Untreated Cells	1417 (1337—1498)
Cells + N	1993 (1845—2142)
Cells + N + 48/80*	2040 (2006—2074)
Cells + 48/80	2919 (2712—3127)
Cells + 48/80 + N**	3024 (3003—3046)

*Treated 10 min 37° C with ninhydrin before adding 48/80
**Treated 60 sec 37° C with 48/80 before adding ninhydrin

$^{22}Na^+$ (mean and range, cpm per 10^6 cells) was 1124 (1092—1156) and 1567 (1461—1674) respectively This shows that in normal cells ouabain alone produces a marked increase in the net $^{22}Na^+$ uptake

b) Fig 6 shows the net uptake of $^{22}Na^+$ in mast cells treated with compound 48/80 (1 µg/ml) in the presence or absence of ouabain (10^{-4} M) The maximal uptake recorded in either case is almost identical but with prolonged incubation times the inhibitory effect of ouabain on the expulsion of ^{22}Na from the cells is reflected in the consistently higher levels of ^{22}Na found in the ouabain treated cells

Comparison of the effects of compound 48/80 and other histamine liberators on net ^{22}Na uptake by mast cells in vitro

The ^{22}Na uptake by normal mast cells cells treated with compound 48/80 (1 µg/ml) and cells treated with two other histamine releasers was compared (Table V) Frisk Holmberg and Uvnas (1968) have recently shown that d tubocurarine in the

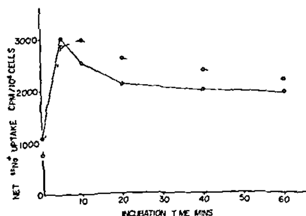


Fig 6 Net ^{22}Na uptake by mast cells exposed to compound 48/80 (1 µg/ml) in the presence (broken line) or absence (full line) of ouabain (10^{-4} M) Single experiment

TABLE V. Comparison of the effects of compound 48/80 and other histamine releasers on net ^{22}Na uptake by mast cells. Means of duplicates and range

Net ^{22}Na Uptake (cpm/10^6 cells)					
Normal	48/80 (1 µg/ml)	d Tubocurarine	(Conc)	n Decylamine	(Conc)
800 (738—862)	4319 (4151—4488)	2564 (2551—2577)	(5×10^{-4} M)	1049 (1044—1055)	(1.6×10^{-4} M)
		3163 (3127—3200)	(10^{-3} M)	364 (364—364)	(3.2×10^{-4} M)
577 (575—580)	1721 (1679—1764)	1620 (1604—1637)	(5×10^{-4} M)		
		1783 (1712—1854)	(10^{-3} M)		
66 (642—692)	4410 (4194—4627)			176 (129—224)	(3.2×10^{-4} M)

concentrations used here (5×10^{-4} M and 10^{-3} M) produces a (20 % and 34 % respectively) release of histamine from mast cells isolated in the same way as those in these studies. Exposure of the cells to d tubocurarine produced a marked increase in net ^{22}Na uptake. According to Bloom and Haegermark (1967) n-decylamine in a concentration of 3.2×10^{-4} M causes a 93 % release of histamine, 1.9×10^{-4} M releases 26 % and 1.3×10^{-4} M 6 %. The lower concentration (1.6×10^{-4}) of n-decylamine had little effect on the net ^{22}Na uptake but the higher concentration we used (3.2×10^{-4} M) produced a lower ^{22}Na uptake than in untreated cells.

Calculation of the quantity of sodium taken up by mast cells

1 ml of the incubation medium contains approximately 3 100 µg of ^{23}Na. The quantity of ^{22}Na is negligible.

In eleven experiments each with an incubation time of 20 min at 37° C followed by the general washing procedure the mean net ^{22}Na uptake by normal mast cells was 716 cpm/10^6 cells, the mean uptake in compound 48/80 (1 µg/ml) treated cells after 20 min was 3 016 cpm/10^6 cells. These figures correspond to approximately 0.01 % and 0.04 % respectively of the total radioactivity (and therefore ^{22}Na) in the medium. Assuming that ^{23}Na and ^{22}Na are taken up at the same rate and to the same extent, the total uptake of ^{23}Na by normal cells would be 0.3 µg/10^6 cells and by 48/80 treated cells it would be 1.2 µg/10^6 cells. If one atom of sodium replaces one molecule of histamine then 0.3 µg would suffice to replace 1.5 µg of histamine and 1.2 µg would replace 6.0 µg of histamine. The quantity of sodium taken up within 30 sec of exposure to compound 48/80—the time period sufficient for histamine release to be completed—was less than 0.5 µg, sufficient to release only about 2.4 µg of histamine.

Discussion

When mast cells are exposed to compound 48/80 *in vitro* they respond by extruding a proportion of their granules. Most of these granules adhere to the outside of the cells giving them a "mulberry-like" appearance (see photomicrographs by Uvnas and Thon 1961). If our 48/80-treated cells were centrifuged and the radioactivity (^{22}Na) in the cells was measured directly this would give an inaccurate value for the *cellular* uptake of ^{22}Na, since a considerable part of the radioactivity would stem from contaminating incubation fluid (with a high level of ^{22}Na activity) and from ^{22}Na attached to the extruded granules still adhering to the degranulated cells. However, when the 48/80-treated cells were washed several times in the buffered salt solution (containing no ^{22}Na) both of these sources of contamination were eliminated or at least greatly reduced by a) dilution of the radioactivity in the suspension medium, b) exchange of ^{22}Na in the extruded granules for ^{23}Na in the washing fluid and c) physical removal of adhering granules—such granules are not precipitated by the centrifugation ($350 \times g$) used for the collection of cells in the washing procedure. Since the washings were carried out at 0° C there should be very little membrane transport, either active or passive, so losses of *cellular* ^{22}Na should be minimal. The recorded radioactivity of the washed mast cells therefore probably reflects fairly accurately the net uptake of ^{22}Na from the incubation medium.

The increased sodium uptake in mast cells exposed to compound 48/80 begins after a latent period of 5—10 seconds, about the same delay as for the degranulation and histamine release caused by this agent. However, the sodium uptake induced by compound 48/80 continues and reaches a maximum after about 10 minutes, in marked contrast to the histamine release which is completed within 20—30 secs at 37° C (Uvnas, Moran and Westerholm 1962; Uvnas 1963; Bloom, Fredholm and Haegermark 1967). If sodium uptake by the mast cells was required for an intracellular exchange between histamine and sodium in the granules then the uptake of sodium should be expected to precede the release of histamine and to reach its maximum before the histamine release was complete. The present observations therefore present strong evidence that ^{22}Na uptake and histamine release are two dissociated processes, even if both are secondary to the action of compound 48/80 on the mast cells.

In addition, from a quantitative point of view, the uptake of sodium was insufficient to explain the histamine release caused by compound 48/80 as the result of an intracellular ion exchange between sodium and histamine. Under the incubation conditions used 1 μg/ml of compound 48/80 causes the release of ca 7—10 μg of histamine per 10^{6} mast cells. The quantity of sodium taken up within 30 sec of exposure to compound 48/80—the time period sufficient for the histamine release to be completed—was less than 0.5 μg, sufficient to release only about 2.4 μg of histamine.

Although the degranulation and histamine release following the exposure of mast cells to compound 48/80 are not dependent on the uptake of sodium, all these effects seem to be the result of enzymatic and not purely physical changes triggered by this releaser. Thus these effects are related to the concentration of compound 48/80 used

and are inhibited at low (0° C) or high (46° C) temperatures and by N ethyl maleimide or ninhydrin

Mast cells which have already reacted once on exposure to compound 48/80 will respond again with degranulation and histamine release to a second challenge with the releaser provided that they are washed between the two exposures in salt solutions not containing compound 48/80 (Uvnas and Thon 1967) However if the mast cells are exposed to a second dose of compound 48/80 *without* any intervening wash degranulation and histamine release do not take place an observation confirmed by us (in Table II 43 % histamine release after one and 44 % total release after two exposures *i e* negligible response to the second exposure) However an increase in the ^{22}Na uptake still occurs after the second exposure even if the response is somewhat lower than that to the first exposure (Fig 5) With the technique used this ^{22}Na uptake in response to the second exposure is only observed when ^{22}Na is added *after* the first exposure to the releaser (Fig 5) If the ^{22}Na is added before the first exposure (Table II) then there is no apparent ^{22}Na uptake in response to the second exposure (*i e* the total net ^{22}Na uptake for the two exposures is not greater than after the first exposure alone) This is probably due to the fact that after the first exposure the cells are loaded with ^{22}Na and on the second exposure there is an exchange of ^{22}Na in the cells for ^{23}Na in the medium masking the sodium uptake

From these observations it seems that ^{22}Na uptake is secondary to some initial changes produced by compound 48/80 in the permeability of the mast cell membrane These changes are normally followed by a chain of events leading to degranulation and histamine release but for some reason these events do not take place in cells exposed a second time to compound 48/80 without an intervening wash

We would expect compounds producing degranulation and histamine release by similar mechanisms to produce similar changes in ^{22}Na uptake Recent work by Frisk Holmberg and Uvnas (1968) has shown that d tubocurarine releases histamine from mast cells in a similar way to compound 48/80 The release involves degranulation is inhibited by similar inhibitors and is temperature dependent in the same way as the release due to the latter substance The ^{22}Na uptake results obtained are similar for both compounds

Bloom Fredholm and Haegermark (1967) showed that the effects of n decylamine on mast cell morphology were different from those produced by compound 48/80 After exposure to n decylamine (3.2×10^{-4} M) the intergranular cytoplasm was grossly affected and only remains of cytoplasmic membranes and organelles were seen Furthermore the cell membrane was disrupted and the cytoplasmic structures appeared to be freely exposed to the extracellular medium This would explain the low net ^{22}Na uptake seen on exposure of the cells to 3.2×10^{-4} M of n decylamine The altered cells will initially take up ^{22}Na from the incubation medium but this will be removed by exchange for ^{23}Na in the washes as in the case of the frozen and thawed cells The lower concentration of n decylamine (1.6×10^{-4} M) had little effect on either ^{22}Na uptake or histamine release (Bloom and Haegermark 1967)

The result of the studies with ouabain suggest that the mast cells contain a mechanism ('sodium pump') which normally removes sodium from the cell. Incubations in the presence of ouabain resulted in an increased net ^{22}Na uptake in both normal cells and cells treated with compound 48/80, probably due to a decrease in the removal of sodium from the cell in the presence of this sodium pump inhibitor.

We conclude from the present study that the increased $^{22}Na^+$ uptake in mast cells after exposure to compound 48/80 is the result of enzymatic changes triggered by this releaser. However, these results do not support the theory that histamine release is brought about by an intracellular exchange of sodium ions for histamine. Thus of the two alternative hypotheses—extra- and intracellular cation exchange—which have been proposed (*vide* Introduction) the former appears to have more experimental support.

This investigation was supported by NIH grant 2ROI AM 04063 08 and Swedish Medical Research Council grant B69 14X 39 05A.

References

ÅBORG, C. H. and B. UVNÄS, Mode of binding of histamine and some other biogenic amines to a protamine heparin complex *in vitro*. *Acta physiol. scand.* 1968. *74*. 552—567.

ÅBORG, C. H., J. NOVOTNY and B. UVNÄS, Ionic binding of histamine in mast cell granules. *Acta physiol. scand.* 1967. *69*. 276—283.

BLOOM, G. D. and Ö. HAEGERMARK, Studies on morphological changes and histamine release induced by bee venom, n-decylamine and hypotonic solutions in rat peritoneal mast cells. *Acta physiol. scand.* 1967. *71*. 257—269.

FREDHOLM, B. and Ö. HAEGERMARK, Histamine release from rat mast cells induced by a mast cell degranulating fraction in bee venom. *Acta physiol. scand.* 1967. *69*. 304—312.

FRISK-HOLMBERG, M. and B. UVNÄS, The mechanism of histamine release from isolated rat peritoneal mast cells induced by d-tubocurarine. *Acta physiol. scand.* 1969. *76*. In press.

MORAN, N. C., B. UVNÄS and B. WESTERHOLM, Release of 5-hydroxytryptamine and histamine from rat mast cells. *Acta physiol. scand.* 1962. *56*. 26—41.

SHORE, P. A., A. BURKHALTER and V. H. COHN, Jr., A method for the fluorometric assay of histamine in tissues. *J. Pharmacol. exp. Ther.* 1959. *127*. 182—186.

THON, I. L. and B. UVNÄS, Mode of storage of histamine in mast cells. *Acta physiol. scand.* 1966. *67*. 455—470.

THON, I. L. and B. UVNÄS, Degranulation and histamine release, two consecutive steps in the response of rat mast cells to compound 48/80. *Acta physiol. scand.* 1967. *71*. 303—315.

UVNÄS, B., Mechanism of histamine release in mast cells. *Ann. N.Y. Acad. Sci.* 1963. *103*. 278—283.

UVNÄS, B. and I. L. THON, Evidence for enzymatic histamine release from isolated rat mast cells. *Exp. Cell Res.* 1961. *23*. 45—57.

UVNÄS, B. and I. L. THON, A physico-chemical model of histamine release from mast cells. In *Mechanisms of Release of Biogenic Amines*. Pergamon Press. 1966. 361—369.

Acta physiol scand 1969 76 213—235

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Proximal Tubular Reabsorption in the Rat Kidney as Studied by the Occlusion Time and Lissamine Green Transit Time Technique

By

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Received 28 August 1968

Abstract

Bojesen E and P P Leyssac. *Proximal tubular reabsorption in the rat kidney as studied by the occlusion time and lissamine green transit time technique* Acta physiol scand 1969 76 213—235

Proximal reabsorption rate determined as the reciprocal of the occlusion time (1/OT) varied in direct proportion to the inulin clearance (C_{in}) in the range between 1 6 and 0 7 ml/min g KW in nondiuretic rats Within this range transit time varied from 10 to 24 sec in direct proportion to the occlusion time the OT/TT ratio averaged 1 10±0 1 (SD) Proximal intra tubular pressure averaged 12 4±0 8 mm Hg and did not correlate with C_{in}

Below inulin clearance of 0 7 ml/min g KW transit time (TT) was further increasing from 24 to 44 sec while the OT remained unchanged thus the OT/TT ratio was decreasing Proximal intratubular pressure averaged 11 5±0 3 mm Hg

Previous estimates of proximal luminal radius from photomicrographs are revised and a more correct value of about 14 μ was found The length of the proximal segment as measured in microdissected nephrons was 10 4 and 10 5 mm in superficial and juxtamedullary nephrons respectively Reflux from more distal segments represented an error of no more than 12 % in estimation of proximal reabsorption rate from the measured OT

The relationship between OT TT linear velocity of flow and tubular fluid/plasma inulin ratio F/P_{in} at 60 % proximal length are derived in quantitative terms $OT/TT = 1/\ln(F/P_{in})$ From the corrected OT/TT ratio the F/P_{in} ratio was calculated from values obtained in control rats ("state A) in rats with partial constriction of the renal artery ("State B) and in rats with elevated intratubular pressure and urine flow ("State C"

There is a direct proportionality (or balance between the glomerular filtration rate (GFR) and overall tubular reabsorption rate in nondiuretic mammals as apparent from the fact that GFR may vary 100—200 times as much as and independent of variations in urine flow rate Because of the large fraction of filtrate reabsorbed by the proximal tubules (about 75 % it has been generally assumed that GFR and proximal reabsorption rate vary in parallel under such conditions, this would also seem evident considering the hydrodynamics of the nephron particularly in view of

the high resistance segment of the thin limb of Henle's loop Accordingly no correlation between clearances of inulin (C_{in}) and late proximal tubular fluid/plasma inulin ratios (F/P_{in}) in control nondiuretic rats has been reported

The reciprocal of the proximal luminal occlusion time (OT) following abrupt clamping of the aorta above the renal arteries has been introduced as an estimate of proximal reabsorption rate under conditions of equal, or known, luminal cross-sectional area (Leyssac 1963) The measured OT was in fair agreement with that calculated from the known F/P_{in}, the clearance of inulin, and the dimensions and number of nephrons over a more than 2 fold range of variation in the clearance This agreement implies that the rate of reabsorption is independent of the luminal radius (or load")

Using the occlusion time method in studies on nondiuretic rats the rate of proximal reabsorption of salt and water has been shown to vary in direct proportion to the glomerular filtration rate over a range of inulin clearances from 1 7 to 0 7 ml/min g kidney weight (KW), as expected Below this level proximal reabsorption rate appeared to reach a fixed minimum (Leyssac *1963*, *1964* a, Leyssac and Gottschalk 1968) The occlusion time will depend not only upon the reabsorption rate but also upon the luminal volume per unit length at the moment of aortic clamping Since 1/OT was found to vary in direct proportion to the inulin clearance, one would expect the luminal diameter to be unchanged within the range of variation investigated Direct measurements by different techniques of proximal luminal diameters under similar experimental conditions confirmed that the diameter remained unchanged over these spontaneous variations, thereby supporting the validity of the OT method as a measure of reabsorption rate, and indicating again that the rate of proximal reabsorption is independent of the radius (distension) (Leyssac 1963, Baines *et al* 1966) Below C_{in} values of 0 7 ml/min g KW the occlusion time remained unchanged with decreasing clearances, suggesting that the proximal fractional reabsorption should be increasing with decreasing clearance below this level Direct measurements of late proximal F/P_{in} ratios at such low clearance values confirmed this prediction (Leyssac and Gottschalk 1968)

According to the above mentioned observations the rates of filtration and proximal reabsorption vary in parallel in nondiuretic rats over a 2 5 fold range of spontaneous variation without detectable changes in luminal diameter Consequently one would predict that the time for a non-reabsorbed particle to pass the distance from the glomerulus to the last visible convolution of a proximal tubule (usually denoted proximal passage or transit time (TT)) would vary in inverse proportion to the inulin clearance, or in other words would vary in direct proportion to the occlusion time The present experiments were designed to test this prediction

Moreover, based on the fundamental and experimentally supported assumption implied in the OT-method *i e* that proximal reabsorptive capacity is independent of the luminal radius, the relationships between OT, TT, *linear velocity* (*H*) and fractional reabsorption (F/P_{in}) have been derived in quantitative terms Calculations of absolute values of OT TT and F/P_{in} ratios, to be compared with those

measured directly, requires knowledge of the correct absolute value of the proximal luminal radius, results obtained from histological sections of snap-frozen, freeze substituted kidneys (Leyssac 1963) and photomicrographs taken of the kidney surface in vivo (Steinhausen *et al* 1963, Baines *et al* 1966) apparently differed considerably with regard to the absolute value The reason for this discrepancy has been explored and the more likely correct value, inferred from this study, has been used in the calculations

Finally, after the introduction in our laboratory of the lissamine green transit time technique it was observed that some reflux of luminal fluid from the pars recta into the visible convoluted part of the proximal tubule occurred following aortic clamping, *i e* during the occlusion time measurement A similar finding has most recently been reported by Brandis *et al* (1967) Such reflux of tubular fluid represents an error in the quantitative estimate of proximal reabsorption rate from the measured OT Therefore experiments were performed to estimate quantitatively the volume of fluid passing retrograde into the convoluted part of the proximal tubule during the luminal occlusion period The results indicated that the reflux at most would be responsible for a prolongation of the OT of some 12—13 % and that the percentage 'error from this source was independent of the actual value of the OT A close quantitative agreement was found between calculated and directly measured values *of C_{in} and F/P_{in} TT, corrected OT and luminal radius, respectively This agreement* adds further strong evidence in favor of the basic assumption underlying these calculations

Methods

Male white rats weighing about 250 g allowed free access to food and water prior to the experiment were anesthetized by intraperitoneal injection of sodium amobarbital (Amytal), 25 mg/kg body weight (BW) and prepared for occlusion time and micropuncture measure [illegible] 1963 1964 The left femoral and jugular veins were [illegible] jections of lissamine green respectively [illegible] ft intact A plastic cup for holding the [illegible] given intravenously during the time of *preparation usually within 30 min* [illegible] ming dose of 15 mg [illegible] 1 % inulin solution [illegible] jection and the first [illegible] fore and after each clearance period which included two to four serial 5—20 min urine collections The clearance value *reported for each period is the average obtained from these multiple collections Twenty* five units of heparin were injected i v before the first blood sample was drawn Urine was collected directly into calibrated polyethylene tubing and the volume excreted determined from the length of the urine column Inulin in plasma and urine was measured by the method of Bojesen (1957) modified for microanalysis

Occlusion time (OT) and lissamine green transit time (TT) were measured successively immediately after the completion of the last urine collection for clearance measurements If clearance was not measured OT and TT measurements followed a period in which urine flow had been measured and found stable OT was measured as described previously (Leyssac 1964 a TT was measured in the modification of Steinhausen s method (1963) as described by Gertz *et al* (1965) *OT and TT were measured by two experimentators (PPL and AH)* who alternately measured OT and TT without knowing the result of the other In Fig 1 eighteen of the values of OT and fourteen of the values of TT are average values of paired measurements by the two investigators Paired measurements of the OT agreed within 2 sec in

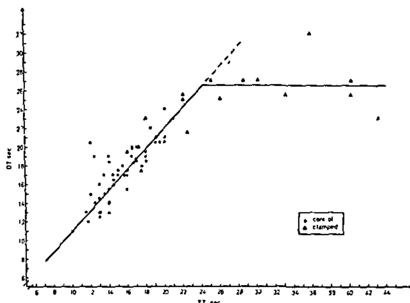


Fig 1 Relationships between Lissamine green transit time and luminal occlusion time Control data represent spontaneous variations

all but one experiment, in which they agreed within 3 sec Paired measurements of TT all greed within 2 sec

Proximal intratubular pressures were measured as described by Gottschalk and Mylle (1956) and registered electronically using a Tybjærg Hansen condensor manometer connected to a high frequency transducer and amplifier (DISA) Intratubular pressures were measured in the period immediately preceding the OT—TT measurements *i e* during the urine collections when inulin clearance was measured, pressures were obtained from several tubules in each period (av 7 7, range 2—13) and the pressure for each period is an average of these multiple measurements Localization of the puncture site to a proximal segment was determined by the later appearance of injected dye in a distal tubule and/or by subsequent localization of the site of puncture in the microdissected nephron

Experiments on reflux of luminal fluid into the proximal convoluted segment during luminal occlusion were performed in the following way Lissamine green (50 μl of a 10 % solution) was injected i v as for transit time measurements The TT was noted At the very moment when the dye had or had almost disappeared from the last visible convolutions the aorta was clamped Dye reflux into one or more proximal convolutions was observed visually and/or photographically by micropuncture a small amount of dye (nigrosin) was injected into the earliest convolution colored by the reflux during the preceding occlusion A second micropuncture in the corresponding (black colored) last visible convolution was subsequently performed and the nephron was filled with dye for identification The kidney was then removed and macerated in hydrochloric acid The sites of puncture was localized in the dissected nephron and the distance between the two puncture sites were measured either with an ocular micrometer and/or in photomicrographs of the isolated nephron (large magnification using a rotameter These two methods gave similar results The distance between the puncture sites was related to the measured total length as well as the length of the convoluted part of the proximal tubule

Photomicrographs of the renal surface (in vivo) and the microdissected isolated nephron were taken through the side arm ... microscope using a Reichert Photoautomatic camera (black ...

A tubular model was made ... for studies of refractive phenomena in tubular ... d illuminated with incident light

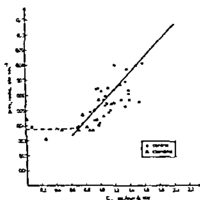


Fig 2

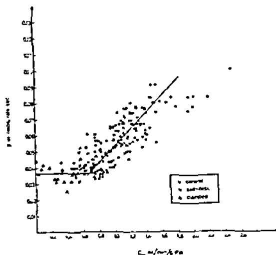


Fig 3

Fig 2 Relationship between inulin clearance and proximal reabsorption rate determined as the [illegible]

Fig 3 Relationship between inulin clearance and proximal reabsorption rate (1/OT) Mass plot of data obtained in the present experiments and previously reported data (Leyssac 1963, 1965 Leyssac and Gottschalk 1968)

Results

A Occlusion time and transit time experiments

1) *Nondiuretic control conditions* Occlusion time and transit time were measured in 50 expts under nondiuretic control conditions 1 to 3 expts were obtained in each single rat The results are presented in Fig 1 There was a linear relationship between OT and TT in the range of OT between 10 and 24 sec This range represents spontaneous variations between individual rats as well as spontaneous (*i e* not deliberately provoked) variations in a single rat during the course of an experiment In 48 expts the OT/TT ratio averaged 1 10 ± 0 1 S D (range 0 90—1 36 In 2 expts excluded from the statistical analysis the OT/TT ratio clearly deviated from the group (OT/TT ratios of 1.59 and 1 75 respectively) However the data from these 2 expts were included in Fig 1 because urine flow rates and the relationship between OT and the inulin clearance were within normal ranges and had been stable prior to the measurements Proximal intratubular pressures were not measured, the reason for the deviation from normal thus remains unexplained In another experiment the OT/TT ratio was also higher than normal (1 85) and the OT—C_{in} relationship was within the normal range This experiment was discarded because urine flow had suddenly increased from 4 8 to 9 1 μl min prior to the experimental period thus rendering the clearance measurement less valid Intratubular pressures were not measured, and a transient pressure diuresis during the OT/TT measure-

ments could not be excluded Thus, as was the case also in previous reports from our laboratory, the present control material was selected by excluding data from (or omitting measurements during) periods prior to and during which a change in intratubular pressure was measured or inferred from sudden changes in urine flow Data from such non steady state periods are presented in section A 3

The single OT-measurement was determined with a S D of ± 0 8 sec as estimated from paired measurements by two investigators not knowing the result of the other The single TT-measurement was determined with a S D of 0 5 sec

Inulin clearances were measured in 32 of the 50 control periods and are plotted against 1/OT in Fig 2 Except for one experiment the results agreed with those reported previously (Leyssac 1963 and 1965, Leyssac and Gottschalk 1968) demonstrating a linear relationship between C_{in} and proximal reabsorption rate determined as 1/OT above clearance values of 0 7 ml/min g KW (Fig 3) In one experiment the relationship between C_{in} and 1/OT deviated from the group, the clearance being higher than that corresponding to the OT The experiment was included in control values, however, because the OT/TT ratio and the measured proximal intratubular pressure were within normal ranges

Urine flow rates averaged 4 0 μl/min (range 1 2—11 5 μl/min) and did not correlate with C_{in} U/P inulin ratios averaged 340 (range 100—660)

Proximal intratubular pressures were measured in 38 periods immediately preceding the OT/TT measurements As had been found previously (Gottschalk and Mylle 1956 Leyssac 1964 b) proximal pressures averaged 12 4 $\pm$ 0 8 mm Hg (range 10 7—14 5 mm Hg) No correlation was found between the inulin clearance and the luminal pressure

2) *Renal artery partially constricted* Occlusion time and transit time were also measured in 16 expts in nondiuretic rats while the renal artery was partially constricted by a 0 20 mm Wilson and Byrom silver clip The results are presented in Fig 1 In 14 of the experiments C_{in} as well as proximal intratubular pressures were measured in the period immediately preceding the OT—TT measurements Clearance values were related to 1/OT measured at the end of the collecting period, the results are given in Fig 2 OT/TT ratios (Fig 1) were inside the range observed in control kidneys in 9 expts in which transit time varied from 16 to 25 sec and occlusion time from 17 to 27 sec Clearance values in these 9 expts were all above 0 75 ml/min g KW and their relationship to the OT did not differ from that obtained in control periods (Fig 2) In the 7 expts, in which transit time exceeded 25 sec a proportionality between OT and TT did not exist, with increasing TT the ratio OT/TT was decreasing because the OT remained unchanged at a maximum value of about 26—27 sec The clearances of inulin were 0 72 ml/min g KW or below in 6 of these experiments, decreasing with increasing TT C_{in} was above 0 75 ml/min g KW only in 1 expt, in which the TT was above 25 sec (C_{in} 0 86 ml/min g KW, OT/TT 27/30 = 0 90) The present data on the relationship between C_{in} and 1/OT below clearance values of 0 7 ml/min g KW, thus agreed with those reported previously (Leyssac 1964 a, Leyssac and Gottschalk 1968) and shown in Fig 3

Urine flow rates during partial constriction of the renal artery averaged 1 2 μl/min (range 0 10—3 10) and correlated with C_{in} below clearance values of 0 7 ml/min g KW as has been reported previously (Leyssac 1964 a) U/P inulin ratios averaged 530 (range 330—1080)

Proximal intratubular pressure averaged 12 3 mm Hg ± 0 6 mm Hg S D (range 11 3—13 2 mm Hg) in the 9 expts, in which clearance values were above 0 75 ml/min g KW Below clearances of 0 75 ml/min g KW proximal luminal pressures averaged 11 5 mm Hg ± 0 34 mm Hg S D (range 11 1—11 9 mm Hg), a value not significantly different from the value reported in the range of inulin clearances from 0 7 to 1 7 ml/min g KW in accordance with previous findings (Leyssac 1964 b)

3) *Non-steady state periods* Results from 11 periods following a sudden increase in urine flow were obtained in the course of experiments on 11 nondiuretic rats Measurements of the various parameters were obtained in 10 expts during a preceding control period, in which urine flow rate had been stable The data from these control and 'abnormal' periods are presented in Table I As is apparent OT/TT ratio, proximal luminal pressure and urine flow rates had increased while the U/P_{in} ratio decreased The increase in the OT/TT ratio was due to the combined effect of an increase in OT and a decreased TT

Inulin clearance was measured in 9 of the 11 periods and in 7 corresponding control periods In the "abnormal periods" C_{in} had either increased (3 expts), decreased (2 expts), or remained unchanged (2 expts) The relationship between C_{in} and 1/OT differed in the 'abnormal periods" from that observed in control periods the measured OT was relatively prolonged in 6 of the 9 periods

Dye reflux into the visible proximal convolutions during luminal occlusion extended to the last visible 1—2 convolutions and was not seen proximal to the last 3 convolutions under these conditions of elevated luminal pressure and urine flow Thus it did not differ from that found in the control periods

The moderate increase in proximal intratubular pressure and urine flow might last for more than 1 hr, but usually urine flow as well as the other parameters had returned to control values within 1 hr

B Proximal tubular dimensions

1) *Luminal radius* In transectioned proximal tubules from the outer cortex of snap-frozen freeze-substituted rat kidneys the luminal radius was found to be 13—14 μ under nondiuretic conditions (Leyssac 1963) This value contrasted to a value of about 10—11 μ measured in photomicrographs of the surface of similarly nondiuretic kidneys (Steinhausen *et al* 1963 Baines *et al* 1966 According to the latter estimate the luminal fluid volume should be only some 60 per cent of that previously calculated from the snap-frozen histological sections In photomicrographs the diameter was measured between the inner borders of the white line of the brushborder—fluid interface (*cf* Baines *et al* Fig 1 This white line about 3—4 μ wide probably represents light reflected from the microvilli Most of the light will pass unreflected through the tubular wall only in the case when the direction of the incident l

Table I Data from nondiuretic rats in control periods with stable urine flow and following a

protocol no	OT (sec)			TT (sec)			OT/TT		
	contr	ab-norm.	Δ	contr	ab-norm	Δ	contr	ab-norm.	Δ
501	12 0	14 5	−2 5	11 5	11 5	0	1 04	1.26	−0 22
502	17 5	22 5	−5 0	15 0	15 0	0	1 17	1 50	−0 33
522	18 5	19 0	−0 5	14 0	11 5	−2 5	1 32	1 66	−0 34
523	22 0	24 0	−2 0	18.5	17 0	−1 5	1 19	1 41	−0 22
524	17 0	20 0	−3 0	16 0	14 0	−2 0	1 06	1 43	−0 37
527	16 5	18 5	−2 0	15 0	13 0	−2 0	1 10	1 42	−0 32
528	—	20 0	—	—	11 0	—	—	1 81	—
mean	17.2	19 8	−2 5	15 0	13 3	−1 3	1 15	1 50	−0 30

rays is more or less parallel to the length of the microvilli. Accordingly the white line (or zone) would represent light reflected from a significant fraction of the inner wall. If this interpretation is correct the distance between the outer rather than the inner borders of the white zone represents the correct value of the luminal diameter That this interpretation is correct could be demonstrated directly in kidneys in vivo by a simple micropuncture experiment. A micropipette containing black dye (nigrosin in saline was introduced in the lumen of a proximal convolution close to the point at which the convolution bends and runs perpendicular to the renal surface Under the conditions of a micropuncture experiment the white zone will be seen broader and more prominent at that part of the lumen which faces the light source. That the lumen is not bordered by the inner aspects of the light zone could be demonstrated by injecting nigrosin into the lumen the rate of injection was adjusted so that dye was flowing slowly out of the tip of the micropipette dye then could be seen to cover and obscure that part of the light zone which was close to the tip This observation shows that the white zone represents light reflection from the lower curvature of the luminal wall.

To illustrate the validity of the above interpretation furthermore photographs were taken of a tubular plexiglass model with an unpolished luminal wall (Fig 4 The model was submerged in water and exposed with incident light imitating conditions in which renal surface tubules were photographed in vivo The distance between the inner borders of the white zone Fig 4 a) obviously gives an erroneously small estimate of the correct luminal diameter (Fig 4 b) On the other hand a little too large



Fig 4 Photograph showing a plexiglass tube embedded in water and exposed with incident light Note the white refractile zone

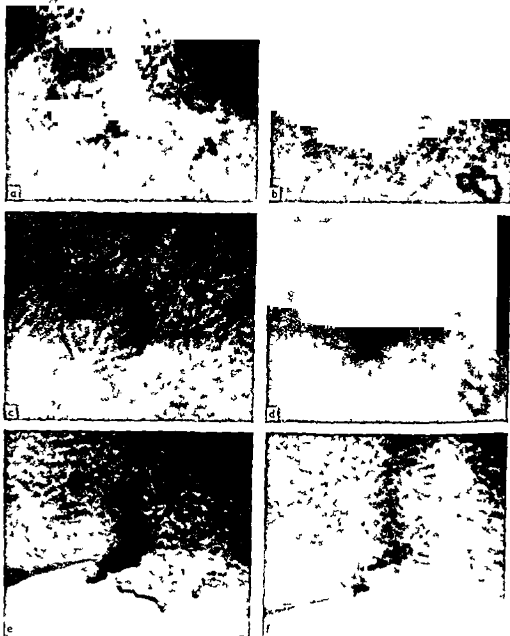
sudden (spontaneous) increase in urine flow (abnormal)

prox intratub pressure (mmHg)			urine flow (μl/min)			U/P in		
contr	ab-norm	Δ	contr	ab-norm	Δ	contr	ab-norm	Δ
13 5	15 2	+1 7	8 6	10 4	+1 8	—	—	—
12 6	22 8	+10 2	4 2	9 3	+ 5 1	350	160	—190
13 2	15 9	+ 2 7	3 2	9 3	+ 6 1	330	110	—220
12 8	15 9	+ 3 1	1 8	14 0	+12.2	530	86	—440
12 7	15 1	+ 2 4	1 8	7 3	+ 5 5	440	89	—351
12 8	14 9	+ 2 1	4 0	7 8	+ 3 8	320	164	—156
—	14 1	—	—	13 0	—	—	109	—
12 9	16 3	+ 3 7	3 9	10.2	+ 5 7	394	119	—271

diameter is measured between the outer borders of the white zone This latter error is due to magnification caused by the large difference between refractive indices of water and plexiglass respectively and the convex outer surface of the tube This error will be insignificant when refractive indices of the two media are much less different such as in the case of tubular fluid versus epithelial cells It is concluded therefore that the distance between the outer borders of the white zone in photomicrographs of proximal tubules represents a more correct value of the luminal diameter than that measured between the inner borders

Luminal diameters were re measured in the original photomicrographs enlarged to a final magnification of 300× (Baines *et al* Fig 1) using the outer borders of the white zone a radius of 13—14 μ was found in close agreement with that measured in histological sections of snap-frozen kidneys A proximal luminal radius of 13—14 μ is therefore considered the most likely correct value under the present non diuretic conditions

2) *Proximal length* The length of the proximal segment from the glomerulus to the beginning of the thin limb of Henle s loop was measured in isolated microdissected nephrons Using an ocular micrometer the proximal length of 13 superficial nephrons averaged 9 35 mm ± 0 56 mm (S D) Proximal length of 12 juxtamedullary nephrons averaged 9 45 ± 0 59 mm (S D) These values represent an underestimate of the true absolute length since it was not possible to stretch the proximal segment completely Comparison of the values obtained in enlarged photomicrographs of the same dissected nephron using the linear distance from the glomerulus to the loop of Henle (as when using the micrometer) and the total distance as measured by a rotameter gave a ratio total length/linear distance of 1 11 Multiplying this correction factor by the measured average proximal lengths the corrected mean values of superficial and juxtamedullary proximal tubules were 10 4 mm and 10 5 mm respectively *The difference is not significant*



F g 5 Se al pho om c ograpl s taken af e the i nje on of L sam ne g ee /e o me def ned by tl e appearance of L g n pentul ular cap lla e a Expo ed at 15 sec lows dye n late v s ble con olu ons b 19 ec Show d e conf ned to last s ble con olu on c 3 ec Dye has d sappeared from s ble par of p ox mal con ol t ons Tl e ao ta wa cla nped at 2? sec d 37 sec sl ow ng dye eflux to la t le on olu on e F rst n cro nj nto he n o t prox mal con olut on colored by L g efl x f Second m cro nj n o the la t a le on ol t on of the same nephron
For further deta ls see tl e tex

C Reflux studies

The volume of fluid flowing retrograde into the convoluted part of proximal tubules during the luminal occlusion following aortic clamping was studied in 15 expts Serial photomicrographs of a representative experiment are shown in Fig 5 Lissamine green (L g) appeared in the capillaries (time zero) about 3 sec after start of the i v injection A green fluid column then was seen to proceed from early to late proximal convolutions, the last visible proximal convolution of 3—4 nephrons usually converged before disappearing as a bundle of partes rectae into the inner cortex and medulla as described by Gertz *et al* (1965) and shown in Fig 5 a, transit time was measured at the moment when dye appeared in the last visible convolution of a group of nephrons, and was 14 sec in the experiment shown in the figure The aorta was clamped at the moment when dye disappeared from the last convolution (at 22 sec in the experiment illustrated in Fig 5) Thus, at the moment of clamping dye was present in the earliest part of pars recta and further downstream Dye reflux into the last visible convolution was not seen until 5—7 sec after clamping but it continued until the end of the occlusion period When luminal occlusion was complete (at 38 sec in the experiment shown in Fig 5 OT − 16 sec) dye was usually present in the last visible 2 convolutions (Fig 5 d) and was never seen proximal to the 3 last convolutions The distance between the site of puncture in the most proximal convolution colored by reflux (Fig 5 e) and the puncture site in the last visible convolution (Fig 5 f) was subsequently measured in the microdissected nephron (Fig 6) and was related to the total length of the proximal tubule Reflux extended to an average of 14 % ± 3 % S D of the total length This correspond to an average of 23 4 % of the length of the convoluted part since the latter amounted to 60 % of the total proximal length The extent of reflux was unrelated to the actual value of the OT and TT over a range of occlusion times from 12 to 26 sec

The luminal diameter is visibly reduced at the moment of initiation of reflux (5—7 sec after clamping) and is decreasing to zero in the following 10—15 sec an approximate estimate of the volume flowing retrograde therefore must be calculated from the length of reflux and an estimated average luminal cross sectional area during the reflux period A volume corresponding to 12 % or less of the total volume



Fig 6 The same nephron as shown in Fig 5 Photomicrograph of the microdissected proximal segment showing the two puncture sites the second in the last visible convolution

Table II. Functional states of the proximal system in nondiuretic rats (BW 200—300 g). The
in x, all other factors remaining constant. k' and k'' (in state A) represent correlation constants

Characteristics		State A	
class	individual	*A state in which control is achieved* Stimulus normal	
primary	f = contributing fraction of total number of nephrons	$f = 1$	
	C_{in} = inulin clearance (ml/min g KW)	$0.7 < C_{in} < 1.8$	
	p = intratub pressure (mm Hg)	$p = 12–13$	
	r = luminal radius (μ)	$r = 14$	
		theoretical prediction	experimental observation
derived	F/P inulin ratio at 60 % prox length	$\frac{\delta F/P}{\delta C_{in}} = 0$	$F/P = 2.8$ independent of C_{in} (ref. a)
	1/OT reciprocal proximal luminal occlusion time (sec^{-1})	$\frac{\delta 1/OT}{\delta C_{in}} = k'$ ($k > 0$)	*1/OT proportional to C_{in}* (cf. fig. 3)
	1/TT reciprocal transit time through 60 % length (sec^{-1})	$\frac{\delta 1/TT}{\delta C_{in}} = k''$ ($k > 0$)	1/TT proportional to C_{in} (cf. figs. 1 & 2)
	OT/TT	$\frac{\delta\, OT/TT}{\delta C_{in}} = 0$	OT/TT = 0.95 for all values of C_{in} (cf. fig. 1 and p. 21)
	U = urine flow (μl/min)	$\frac{\delta U}{\delta C_{in}} = 0$	U uncorrelated to C_{in} (ref. c)

present in the convoluted visible segment at the moment of aortic clamping, thus, is considered to be a maximum estimate of the retrograde flow under the present conditions.

Discussion

I. *Theoretical considerations*

The introduction of the occlusion time method for evaluating variations in proximal reabsorption rate under conditions of equal luminal cross-sectional area was based on the assumption that the reabsorptive capacity primarily is independent of the "load" as such, the vague term "load" representing parameters such as amount (= vol. x conc. of solutes), pressure or flow rate. In this case proximal reabsorption rate

formalism $\frac{\delta y}{\delta x}$ is used to express the variation in the parameter y as a function of increments Ref. a Gottschalk and Leyssac 1968 Ref b Cortney *et al* 1965 Ref c Leyssac 1964 b

State B		State C	
A state in which control is not achieved Stimulus supramaximal		A state in which control is not achieved Stimulus abnormal or ineffective	
$f < 1$ $0.2 < C_{in} < 0.7$ } correlated $p = 10$—13 $r = 14$		$f = 1$ $0.7 < C_{in} < 1.8$ $13 < p < 16$ } uncorrelated $r = 14$	
theoretical prediction	experimental observation	theoretical prediction	experimental observation
$\frac{\delta F/P}{\delta C_{in}} < 0$	F/P 5.5→2.8 for C_{in} 0.2→0.7 (ref a)	$\frac{\delta F/P}{\delta p} < 0$	isotonic loadings F/P 2.8→2.0 (ref b)
$\frac{\delta 1/OT}{\delta C_{in}}$ 0	1/OT constant for $0.3 < C_{in} < 0.7$ (cf fig 3)	$\frac{\delta 1/OT}{\delta p}$ 0	1/OT 0.058→0.051 for p 13→16 (table I)
$\frac{\delta 1/TT}{\delta C_{in}}$ 0	1/TT 0.025→0.05 for C_{in} 0.25→0.7 (cf figs 1 & 2)	$\frac{\delta 1/TT}{\delta p} > 0$	1/TT 0.067→0.075 for p 13→16 (table I)
$\frac{\delta OT/TT}{\delta C_{in}}$ 0 OT/TT 0.95	OT/TT 0.65→0.95 for C_{in} 0.2→0.7 (*cf figs 1 & 2*)	$\frac{\delta OT/TT}{\delta p} > 0$	OT/TT 0.95→1.31 for p 13→16 (*table I*)
$\frac{\delta U}{\delta C_{in}}$ 0	positive correlation (ref c)	$\frac{\delta U}{\delta p}$ 0	U 3.9→10.2 for p 13→16 (table I)

per unit of tubular length calculated from the luminal cross-sectional area and the measured occlusion time (*i e* during the occlusion period in which filtration is interrupted equals the rates of reabsorption in the free flow periods immediately preceding and following the occlusion period Consequently OT should be quantitatively related to other parameters dependent on the rate of reabsorption such as transit time as well as F/P_{in} ratio- and inulin clearance measured in the free flow condition immediately prior to or after the occlusion time measurement

Results from investigations testing the occlusion time method have provided a considerable amount of evidence supporting the validity of the OT as a method for evaluating variations in proximal reabsorption rate under conditions of equal free flow diameters this will justify the main assumption underlying the derivation of the following relationships

(a) $$C = \frac{\pi r^2}{OT} \quad \text{(C independent of r)}$$

in which C is proximal reabsorptive capacity per unit of tubular length, r is luminal radius and OT is the luminal occlusion time

The further assumptions have been made

(b) luminal radius is constant along the convoluted part of the proximal tubule

(c) C is constant along the convoluted part of the proximal segment, this is considered a reasonable approximation because 1) the OT is well defined, i e early and late proximal con

rapid removal (due to reabsorption) of the fluid layer lining the luminal wall

From these assumptions the following equations have been derived

If volume flow rate at a certain distance (l) from the glomerulus is denoted by V we have

(1) $$-dV = C\,dl = \frac{\pi r^2}{OT}\,dl \quad (mm^3/sec)$$

thus flow rate decreases linearly with the distance l Since linear velocity, denoted by H equals $\frac{V}{\pi r^2}$ (mm/sec), it follows that

(2) $$-dH = \frac{dl}{OT}$$

Eq (2) integrated from length O to L, and H_0 to H_L yields

(3) $$OT = \frac{L}{H_0 - H_L} \quad (sec)$$

in which L is any given distance along the proximal convoluted segment, and H_0 and H_L are linear velocities at the glomerulus (L=O) and at the distance L from the glomerulus respectively

Since volume flow rate decreases linearly with the length linear velocity also decreases linearly with length, and we have

(4) $$dL = H_L dt = (H_0 - \alpha L)\,dt$$

in which the slope $(\alpha) = \frac{H_0 - H_L}{L}$

Integrated from time O to T_I and length O to L equation (4) yields

(5) $$H_L = H_0 e^{-\alpha T_L}$$

from which we get

(6) $$T_I = \frac{\ln H_0 - \ln H_L}{H_0 - H_L}\,L \quad (sec)$$

If we choose L as the distance from the glomerulus to the last visible convolution i e 60 % proximal length T_I will equal transit time

Rearranging eq (6) we get

(7) $$TT = \frac{L}{H_0 - H_L} \ln \frac{H_0}{H_L} \quad (sec)$$

Combining eqs (3) and (7) we have

(8) $$OT/TT = \frac{1}{\ln\left(\frac{H_0}{H_L}\right)}$$

Since $\frac{V_0}{V_L} = \frac{H_0}{H_L} = \frac{F}{P}$ inulin ratio (at the distance L from the glomerulus) it follows that

(8 a) $$OT/TT = \frac{1}{\ln(F/P_{in})}$$

In Fig 7 the ratio OT/TT is plotted as a *function of F/P_{in}*

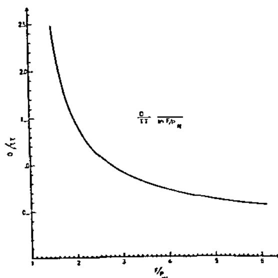


Fig 7 The calculated relationship between the occlusion time/transit time ratio and the proximal tubular fluid/plasma inulin ratio

Rearranging eq (3) and substituting F/P_{In} for $\frac{H_o}{H_L}$ in eqs (3) and (7) we get

$$\text{(3 a)} \qquad OT = \frac{L}{H_o\left(1 - \frac{1}{F/P_{In}}\right)} \text{ (sec)}$$

and

$$\text{(7 a)} \qquad TT = \frac{L}{H_o\left(1 - \frac{1}{F/P_{In}}\right)} \ln F/P_{In} \text{ (sec)}$$

II *Experimental observations*

In the present study we have extended previous investigations by combining the transit time technique with the clearance—and occlusion time methods In addition intratubular pressure was measured The present results indicate that in nondiuretic rats occlusion time and transit time vary in direct proportion over a range of inulin clearances from about 0 6—0 7 to 1 7 ml/min g KW This is in general agreement with predictions from experiments utilizing the occlusion time and clearance methods and the finding of unchanged luminal diameters over a similar range of clearance values in nondiuretic rats The present observations therefore support the validity of previous results

A proximal luminal radius of about 14 μ seems to be the best approximation to the actual value as estimated by three independent methods A value of 14 μ was found by direct measurements of diameters in sections of snap-frozen kidneys in close agreement with those measured from photomicrographs taken in vivo when present observations from the model and in vivo by micropuncture were taken into consideration. The same value is calculated from the amount of draining fluid collected from the renal vein after removal of the kidney (0 20 ml/rat kidney) and the length

and number of proximal tubules (*cf.* discussion by Leyssac 1966, p. 62). In the further quantitative analysis a radius of 14 μ has been used in calculations of proximal luminal volume and linear velocity at the beginning of the tubule (H_0).

The observation of retrograde flow into the proximal convoluted segment during luminal occlusion rendered a correction factor to the occlusion time necessary for quantitative estimates and analyses. Brandis *et al.* (1967) suggested that the reflux would represent about one third of the total proximal fluid volume, *i.e.* would represent an error of about 50 % in the occlusion time, but as the authors admitted their results were qualitative only, and in their estimate they did not take into consideration the reduction in luminal volume during the reflux period. The present results indicate that a correction factor of about 0.88 represents a maximum estimate of the error. Since we found the volume flowing retrograde independent of proximal rate of reabsorption, a correction factor of 0.88 should be applied to all measured values of occlusion time short as well as long (corrected OT 12 % less than that measured). Since it represents a constant percentage for all values of OT, it does not of course, invalidate conclusions drawn from previous results obtained by this method. Using the corrected OT values the quantitative agreement with data obtained by other independent methods is improved in fact as will appear from the following calculations.

Proximal tubular length was the same (10 mm) in superficial and deep nephrons in the present strain of rats. So a priori there are no reasons or evidence to suggest a significant difference either in rate of filtration or proximal reabsorptive capacity per nephron between superficial and deep nephrons. Furthermore because of the shape of the kidney the total volume of deep proximal segments is considerably less than that of superficial proximal segments. Thus even a moderate difference in reabsorptive capacity within the physiological range of variation could not introduce any significant error in the following quantitative analysis.

We may confine the following analysis by way of example to a nondiuretic condition at an inulin clearance (V_a) of 1.0 ml/min g KW (= 0.87 ml/min per kidney). Since a luminal radius of 14 μ appears to be the most likely correct value and the number of nephrons per kidney is 32 000 (Smith 1951), initial linear velocity (at the glomerulus) is

$$H_0 = \frac{0.87 \times 10^9}{\pi\, 14^2 \times 32\,000 \times 60} = 0.74 \text{ mm/sec}$$

Tubular fluid/plasma inulin ratios obtained in several laboratories from the last visible proximal convolutions (L = 60 % total proximal length = 6 mm) averaged about 2.7–2.8. Inserting these values in eq. (3 a) the calculated occlusion time

$$OT_{calc} = \frac{6}{0.74\left(1 - \frac{1}{2.7}\right)} = 13 \text{ sec}$$

When corrected for retrograde flow we have

$$\text{corrected } OT_{meas} = 16 \text{ sec}$$

Directly measured occlusion times at this clearance value averaged 18.5 sec (*cf.* Fig. 3). Inserting in eq. (7 a) the same values

$$TT_{calc} = \frac{6}{0.74\left(1 - \frac{1}{2.7}\right)} \ln 2.7 = 13 \text{ sec}$$

to be compared with that directly measured

$$TT_{meas} = 17 \text{ sec}$$

In the case of occlusion time as well as transit time we consider this a fair agreement, particularly in view of the approximations inherent in the calculation of H_0 and the measurement of L

Expressed in μeqv Na^+ reabsorbed per unit of luminal surface area[1] and time, and using the same values as above and a plasma sodium concentration of 150 μeqv /ml, 7 15×10⁻⁴ μeqv / mm² · sec was calculated from the measured C_{in} and F/P_{in} ratio, to be compared with 6 50×10⁻⁴ μeqv/mm² sec calculated from the corrected occlusion time of 16 sec

Inserting in eq (8 a) the corrected OT_{meas} and the TT_{meas} at an inulin clearance of 1 0 ml/min g KW (16 and 17 sec, respectively) we get

$$\text{corrected } OT/TT = 0\,95 = \frac{1}{\ln (F/T_{in})}$$

[illegible] calculated value [illegible] (as about [illegible] directly at [illegible] experimental [illegible] the present calculated value, whereas the [illegible] sit time are a little larger than those calculated This suggests a minor error(s) in the estimate of a common factor entering with almost equal weight the calculations of the two time periods

In conclusion, the close quantitative agreement obtained between calculated and directly measured values of OT, TT, and C_{in} and F/P_{in} cannot be considered fortuitous because of the complete independence of the methods for measurement of these parameters, therefore it seems to establish the validity of the assumptions upon which the calculations of these parameters were based (and the reliability of the experimental methods) On the other hand measurements of reabsorptive halftime ($t_{1/2}$) using the split-oil drop technique so far have failed to demonstrate any significant correlation with inulin clearance or transit time in normal nondiuretic rats (Gertz *et al* 1965, Rector *et al* 1966, Brunner *et al* 1966), the occlusion time method, therefore, is superior to the split-oil drop technique when applied to this experimental condition

In Table II the present theory and results are given in schematic form The proximal system (glomeruli with affiliated arterioles and the proximal tubules) is considered as a unit defined by a control system, in which the renin angiotensin system probably constitutes the efferent pathway (Leyssac 1966) Three different functional states have been characterized and experimentally investigated The states are defined by the primary characteristics, as enlisted in the table From these primary characteristics five other characterizing parameters have been derived theoretically as well as tested experimentally Under certain conditions a perfect control may be maintained over a wide range of activity levels (filtration-reabsorption rates) so that the load to the more distal segments of the nephron is independent of the level at which the proximal system is set (State A) State B may be provoked by partial constriction of the renal artery or sodium deprivation it represents the functional steady state, in which the capability of the control system in stabilizing the delivery of tubular fluid

[1] Such a calculation is based on the surface area of a smooth cylinder, thus disregarding the presence of microvilli

to more distal segments is surpassed in spite of maximum stimulation. In state C, which may occur spontaneously, the control is disturbed for yet unknown reasons.

As apparent from Table II the theory correctly predicts the direction of changes and the magnitude of the five derived parameters in all three states with only one exception, in state C the premises of primary characteristics predicts invariance of the occlusion time with intraluminal pressure changes, in disagreement with the experimental observation of a relatively prolonged OT. Although the changes in occlusion time were only minor they coincided with consistent changes in intratubular pressure, urine flow and U/P_{in} ratio, and, because the occlusion time invariably increased while the transit time decreased or in only two experiments remained unchanged, the OT/TT ratio was significantly increased in this state. Such consistent pattern makes it highly unlikely that the changes in OT should be interpreted as errors of measurement rather than a characteristic of a different functional state. From the measured mean transit time and mean occlusion time, the latter corrected for reflux, a mean corrected OT/TT ratio of 1.31, corresponding to a F/P_{in} ratio of 2.1 was found during periods with elevated luminal pressure. Thus, proximal fractional reabsorption was reduced by about 13 % from control values. Since no significant changes occurred in inulin clearance, and urine flow increased by less than 1 % of the rate of filtration, it is inferred that the rate of reabsorption per unit of tubular length was decreased (15—20 %) in the proximal tubules and markedly increased in a segment(s) distal to the proximal tubule. The most simple interpretation of the above finding imply that the hydrodynamic resistance to flow in the loop of Henle was much reduced by dilatation possibly secondary to the elevated proximal pressure. In other words the "glomerulo-tubular balance" was disturbed in these periods for unknown reasons. In this interpretation an unchanged distribution of filtration rates between superficial and deep nephrons is assumed.

Gertz *et al.* (1965) have proposed an alternative to the present concept. Gertz theory, which is a modification of the classical concept, postulates that the rate of proximal reabsorption is an automatic function of the luminal cross-sectional area or $C \cdot k \cdot r^2$. Difficulties in attempts to fit this theory to experimental data has made it necessary to postulate in addition "extrinsic" factors modulating the postulated automatic "intrinsic" dependency of the degree of distension (Rector *et al.* 1967). According to this point of view such "extrinsic" factor(s) must have taken over the entire control in state A leaving no room for the hypothetical basic dependency of r^2.

Gertz theory implies that changes in the mechanical working conditions of the proximal cells will influence their rate of net transfer directly. Therefore the effect of changes in r^2 on the proximal reabsorptive capacity should be most clearly and readily demonstrable in acute experiments in which the radius is rapidly changed such as during the occlusion phenomenon. Such an immediate reaction to changes in the degree of luminal distension has also been assumed by Gertz (pers. communication) and apparently by other advocates of his theory (Wiederholt *et al.* 1967, Wahl *et al.* 1967).

However, the present results from occlusion time and transit time measurements as well as previous results obtained by the occlusion time method will appear incompatible with the theory postulating that

$$(9) \qquad -\frac{dV}{dt} = C = k\,r^2,$$

in which V is luminal volume per unit of tubular length

First, according to this theory complete luminal occlusion due to reabsorption following interruption of glomerular filtration should never be accomplished This prediction is inconsistent with the observation that the proximal lumens appear in fact completely occluded

in occlusion time, we may assume a constant OT value equal to the average corrected OT of 16 sec at an average inulin clearance of 1 0—1 1 ml/min g KW Differentiating $V = \pi r^2$ with regard to r and substituting $dV = -kr^2dt$ we have

$$(10) \qquad -\frac{dr}{r} = \frac{k}{2\pi}dt$$

By integration eq (10) yields

$$(11) \qquad -\ln r = \frac{k}{2\pi}t + I,$$

in which I is the integration constant

In other words Gertz' theory implies an exponential decrease in luminal radius with time during the occlusion, integrating over the occlusion time period with $r_0/10$ as an arbitrary endpoint we have

$$(11\ a) \qquad \ln\frac{r}{r/10} = \frac{k}{2\pi}OT$$

from which it follows that

$$(12) \qquad k = \frac{2\,3 \times 2\pi}{OT} = \frac{2\,3 \times 2\pi}{16} = 0\,9$$

Inserting in eq (9) $k = 0\,9$ and $r = 14\ \mu$ proximal reabsorptive capacity equals $1\,76 \times 10^{-4}$ $mm^3/mm\ sec$ with 32 000 nephrons per kidney a total of 2 0 ml/min should be reabsorbed at the end of the visible part of the proximal convolution (6 mm proximal length) during free flow at a rate of filtration of about 1 0 ml/min per kidney, this obviously is a contradiction[1]

Direct observations are thus grossly inconsistent with those predicted from the theory that the reabsorptive capacity is a function of the radius squared whereas a quantitative agreement is found between values measured directly and those calculated according to the theory that reabsorptive capacity has the character of a Tm-process *i e* independent of radius (or load)

Very recently Thurau and his associates (Wahl *et al* 1967) have reported the results of an investigation using the occlusion time method Their results apparently disagreed quantitatively with those reported in previous and the present investiga-

[1] Even inserting a radius of 10 μ it is calculated that at 60 % proximal length complete reabsorption of the total filtered volume should have taken place, which is inconsistent with observed facts

tion from our laboratory, and it was concluded first that the reabsorptive capacity changes linearly with radius during the occlusion period second that the lateral net fluxes of water under free flow, as calculated from the occlusion time will be over estimated by a factor of 2 Without further comment our previous finding of a varying occlusion time was considered an error However, in view of the results reported here and within the frame of the scheme presented in Table II Thurau s data may be interpreted as a fourth state of function in which the luminal radius shows covariation with the activity level of the proximal system (the inulin clearance) However, lack of information about intratubular pressures and urine flow rates as well as mixing of data obtained from animals with greatly different body weights prevent any precise definition of such a state D A standardization solely of the clearance to a fixed kidney weight without standardizing also the measured radii to kidney weight implies that the number and/or length of nephrons increases with increasing kidney weight which is not the case within this range of kidney weights (Sperber 1944) It remains therefore a question to which extent the observed correlation between standardized values of inulin clearance and luminal radii may be explained by a more labile renal function in preparations of young rats causing an accumulation of data from small animals at the low clearance values It is possible though that the rats investigated by Thurau or at least a group of rats with high clearance values have been in such a state D in which the luminal cross sectional area is correlated to the inulin clearance within the single animal and in groups of animals of equal kidney size In this case the observed invariance of the occlusion time over a wide range of standardized clearances of inulin obtained in rats of different size fits perfectly well to the present theory since it has been reported that the correlation between inulin clearance per g of kidney weight and the reciprocal of the occlusion time seen in state A is the same in young and grown up rats (Leyssac 1965 b) Thus we are inclined to infer from the observed lack of correlation between the inulin clearance and the reciprocal occlusion time that the functional state of at least a group of Thurau s animals actually has differed from that of state A B and C the difference being a covariation of the luminal radius The reason(s) for this difference remains obscure of course but may be related to differences in the anesthetic used and/or in the operative technique Thurau approached the kidney from the flank and suspended it in a cup their kidneys were decapsulated the renal artery dissected and freed from surrounding tissue (partial denervation?) and the clamp was placed upon the renal artery itself Further their preloading of the rats with saline was greater than in our experiments (up to 3 ml) It is tempting to suggest that a state D may be closely related to the state C observed in some of our rats the only difference being that state D occurs in decapsulated kidneys under the same conditions which provoke state C when the capsule is left intact

Thurau s results agree with ours in demonstrating that proximal reabsorptive capacity per unit of tubular length as estimated from the occlusion time and the luminal volume per unit of length varies in direct proportion to the rate of filtration Since this was the case under both conditions in one of which the radius remained constant it seems unjustified to conclude that changes in the radius squared

is the *cause* of the observed changes in reabsorptive capacity Their free flow data thus, *cannot support Gertz' hypothesis*

Thurau further tried to investigate the changes in luminal volume as a function of time during the luminal occlusion period by measuring changes in the luminal radius within the first 6 sec of the occlusion period as well as the corresponding occlusion time These data also disagree with predictions from Gertz theory in showing an apparently linear initial decrease in radius with time with an extrapolated intercept with the abscissa (time) at the measured occlusion time It is interesting that the average rate of reabsorption as estimated from the change in radius over the first 5 sec, is in reasonable agreement with that calculated from the corresponding inulin clearance and the known F/P_{in} ratio A linearity throughout the occlusion period was considered evidence that during the occlusion period reabsorptive capacity changes as a function of the radius (but not radius squared) This conclusion is valid, of course, only if the absolute values of radii measured within the initial 5 sec are correct However, the measured radii will be underestimated by a certain value in all the measurements if the diameter is taken as the distance between the inner borders of the white refractile zone This was probably the case in Thurau s investigation, since the problem of measurement was not mentioned and none of their values reached a value of 14 μ, their value of 9—10 μ reported at an average inulin clearance agrees with that measured between the inner borders under the present experimental conditions (Baines *et al* 1966) Using the more correct values of initial radii an extrapolation of the initial slope will intercept with the abscissa at a value far greater than the occlusion time, thus, their corrected data may fit with closer approximation the curve representing constant reabsorptive capacity during the luminal occlusion in agreement with the present conclusions

Whether or not proximal reabsorptive capacity may be influenced directly by distension of an isolated tubular segment such as under the special conditions of the split oil drop experiment or the microperfusion experiments reported by Wiederholt *et al* (1967) remains to be conclusively demonstrated In the split-oil drop experiment conclusive evidence should be provided, first to exclude that the proportional increase in rate of removal of the fluid volume interposed between the oil droplets observed with increased distension might be due to an increased reabsorptive area (due to increased extension of the menisci) rather than an increased reabsorptive capacity

In the microperfusion experiment (Wiederholt *et al* 1967) fluid was infused at various rates into single proximal tubules and recollected from a later proximal convolution The results suggested that while the luminal diameter increased with perfusion rate (V_0) going from 10 to 24 mμl/min reabsorptive capacity increased proportionally This conclusion was inferred from the finding of an unchanged collected perfusate/injected fluid inulin ratio (CP/IP_{in}) of 2 3 at 2000 μ distance from the site of fluid injection (corresponding to 20 % total proximal length) Thus the results were claimed to confirm the theory of Gertz However several aspects of their findings were not readily explained First the remarkably high CP/IP ratio at 2000 μ distance from the site of infusion was explained according to Gertz theory by

the increase in luminal diameter produced by the microperfusion procedure. This explanation appears inconsistent with their statement that at low perfusion rates (10—12 mμl/min) at which equally high inulin ratios were found no significant difference in diameters between perfused and adjacent nonperfused tubules could be observed. Second, in the analysis of their data they postulate that the CP/IP inulin ratio increases linearly with the distance (L) starting at a ratio of 1.0 at a distance of zero. A satisfactory explanation is not given why all regression lines presented in their figures intercept the abscissa at a value (L) of about 500 μ significantly different from the origin (zero length). Third, their interpretation implies a hyperbolic decrease of proximal reabsorption rate along the tubule. This is in disagreement with previous findings obtained under free flow conditions from the same as well as other laboratories (Giebisch *et al.* 1964, Gottschalk and Lassiter 1966), the data obtained under free flow conditions fit with fair agreement the curve representing constant reabsorption rate along the convoluted proximal segment. According to the theory advocated by the authors a hyperbolic decrease in reabsorption rate with distance implies that the radius squared decreases in inverse proportion to the distance. No evidence has been presented to support this implication.

The microperfusion data were not analyzed in view of the alternative theory that proximal reabsorption rate is approximately constant along the convoluted segment and independent of luminal distension. According to this alternative one would expect a more pronounced increase in luminal pressure following elevation of the perfusion rate. The perfused segment including the puncture hole at the perfusion site would distend and leakage of fluid out of the tubule past the pipette might likely occur. Such an error would readily explain the high and invariable CP/IP inulin ratio obtained at 2000 μ distance at the various perfusion rates. In absolute terms a leakage error will be most significant at the high CP/IP ratios, i.e. at a distance of 2000—2500 μ from the site of perfusion. Consequently the data obtained at shorter distances should be more valid. It is interesting that the CP/IP inulin ratio at 1000 μ distance decreased from about 1.6 to 1.2, corresponding to a fractional water reabsorption of 40 % and 20 %, respectively, as the perfusion rate was doubled from 12 to 24 mμl/min. This finding was to be expected in view of the alternative concept. The criterion that leakage did not occur in these experiments would be the documentation that total amounts of inulin infused ($V_0 \times IP$) equalled total amount recollected ($V_L \times CP$) with different perfusion rates and with different rates of recollection at a constant perfusion rate. Such documentation was not presented. In view of the difficulties in explaining their findings according to the theory of Gertz, the likely leakage error which might explain their data, and lack of evidence against this source of error, the paper appears less conclusive. Considering the most valid range of perfused length the data presented appear consistent with the theory postulating that proximal reabsorptive capacity is independent of the luminal radius.

This work was carried out with the technical assistance of Anna Hansen.
We want to express our gratitude to Dr. K. H. Gertz who suggested and inspired the present experiments during his visit to our laboratory and helpfully demonstrated the transit time technique. We are also indebted to Dr. Gertz for his valuable criticism and discussion of the manuscript.

We want to thank stud med Ole Frederiksen for his valuable contribution to the mathe

References

Baines A D, P P Leyssac and C W Gottschalk Proximal luminal volume and fluid reabsorption in the rat kidney Abstracts II Free Communication III int Congr Nephrology Washington D C 1966 *Acta physiol scand* 1968 *74* 440—452

Bojesen E., A method for determination of inulin in plasma and urine *Acta med scand* 1952 Suppl 266 275—282

Cortney M A M Mylle W E Lassiter and C W Gottschalk, Renal tubular transport of water solute and PAH in rats loaded with isotonic saline *Amer J Physiol* 1965 209 1199—1205

Gottschalk C W and W E Lassiter A review of micropuncture studies of salt and water reabsorption in the mammalian nephron *Proc III int Congr Nephrol* Washington D C. 1966 S Karger Basel 1967 *1* 99—109

Gottschalk C W and P P Leyssac Proximal tubular function in rats with low inulin clearance *Acta physiol scand* 1968 *74* 453—464

Leyssac P P Dependence of glomerular filtration rate on proximal tubular reabsorption of salt *Acta physiol scand* 1963 *58* 236—242

Leyssac P P The in vivo effect of angiotensin on the proximal tubular reabsorption of salt in rat kidneys *Acta physiol scand* 1964 a 62 436—448

Leyssac P P The effect of partial clamping of the renal artery on pressures in the proximal and distal tubules and peritubular capillaries in the rat kidney *Acta physiol scand* 1964 b *62* 449—456

Leyssac P P The in vivo effect of angiotensin and noradrenaline on the proximal tubular reabsorption of salt in mammalian kidneys *Acta physiol scand* 1965 *64* 167—175

Leyssac P P The luminal occlusion time of proximal tubules in young rats *Acta physiol scand* 1965 b *64* 176—181

Leyssac P P The regulation of proximal tubular reabsorption in the mammalian kidney *Acta physiol scand* 1966 *70* Suppl 291 Thesis

Rector F C Jr F B Brunner and D W Seldin Mechanism of glomerulotubular balance I Effect of aortic constriction and elevated ureteropelvic pressure on glomerular filtration rate fractional reabsorption transit time and tubular size in the proximal tubule of the rat *J clin Invest* 1966 *45* 590—602

Rector F C Jr. J C Sellman M Martinez Maldonado and D W Seldin The mechanism of suppression of proximal tubular reabsorption by saline infusions *J clin Invest* 1967 *46* 47—56

Smith H W *The Kidney* Oxford Univ Press New York 1951 pp 1049

Sperber I Studies on the mammalian kidney *Zool Bidrag fran Uppsala* 1944 *22* 249—431

Steinhausen M I Iravani G E Schubert and R Taugner Auflichtmikroskopie und Histologie der Tubulusdimensionen bei verschiedene Diuresezustanden *Virchows Arch path Anat* 1963 *336* 503—527

Wahl M W Nagel H Fuchbach and K Thurau On the Application of the occlusion time method for measurements of lateral net fluxes in the proximal convolution of the rat kidney *Pflugers Arch ges Physiol* 1967 *298* 141 153

Wiederholt M K Hierholzer E E Windhager and G Giebisch Microperfusion study of fluid reabsorption in proximal tubules of rat kidneys *Amer J Physiol* 1967 *213* 809—818

Acta physiol. scand. 1969. 76. 236—247

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The Dynamics of the Inotropic Change Produced by Altered Pacing of Rabbit Papillary Muscle

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Received 9 September 1968

Abstract

Edman, K. A. P. and E. Nilsson. *The dynamics of the inotropic change produced by altered pacing of rabbit papillary muscle.* Acta physiol. scand. 1969. 76. 236—247.

The time course of the active state, the force-velocity relationship and the series compliance of rabbit papillary muscles were studied at different inotropic states produced by altered pacing of the muscles within the range 18 to 72 stimulations per minute. The inotropic intervention produced a shift of the force-velocity curve without markedly changing the shape of the curve, *suggesting that* V_{max} (*the capacity to produce motion*), and P_{max} (*the capacity to produce tension*) were affected to the same degree. Intensification of the active state in response to an increase in stimulation frequency was associated with a steeper rise of the active state and an earlier attainment of maximum. The series compliance was unaffected by the inotropic intervention. The results are taken as evidence (1) that the two parameters of the active state, V_{max} and P_{max}, are governed by a common control mechanism in the excitation-contraction coupling and (2) that the inotropic effect is due to altered kinetics of this control function. The nature of the mechanism governing the active state is discussed in relation to the sliding filament theory of muscular contraction.

Evidence was presented in a previous paper (Edman and Nilsson 1968) that the concept of the active state (Hill 1949), providing a common parameter for the ability of the muscle to produce tension (P_{max}) and to shorten actively (V_{max}), is applicable in the analysis of the heart muscle function. It was shown that cardiac active state, unlike the situation in the skeletal muscle, has a slow onset and does not reach a plateau during a contraction cycle (also see Brady 1966, Edman, Grieve and Nilsson 1966, Sonnenblick 1966). The analysis furthermore demonstrated that the course of the active state is not definitely programmed when the contraction starts; both the maximal intensity of the active state and the duration of the activity are critically dependent upon the amount of shortening of the contractile unit during the contraction period. In fact, the intrinsic length changes of the muscle (5—10 per cent of the resting muscle length) taking place during an ordinary isometric contraction are great enough to affect the course of the active state and hence the isometric twitch output to a substantial degree. The influence

of muscle length upon the active state can be adequately controlled, however Thus as demonstrated previously (Edman *et al* 1966, Edman and Nilsson 1968 Brady 1967), it is possible to define the kinetics of the active state in isolated papillary muscles at a constant length of the contractile unit.

It has been the aim of the present investigation to explore the dynamics of the inotropic change that associates an altered pacing of the myocardium Results of earlier work have suggested (Abbott and Mommaerts 1959, Sonnenblick 1962, 1966) that the inotropic effect involves changes of both P_{max} and V_{max} However the precise way in which these quantities are affected relative to each other has not yet been clearly demonstrated In order to settle this point, the force velocity relationship must be analyzed under careful control of both length of the contractile element and time at which the measurements are made during the activity period The present experiments including these control measures, have shown that over a wide range of stimulation frequencies the shape of the force velocity curve remains virtually constant suggesting that V_{max} and P_{max} undergo very nearly parallel changes in response to the inotropic intervention produced This finding provides relevant information for evaluation of the mechanism through which the inotropic effect is brought about

Methods

Isolated papillary muscles of the rabbit were used The rabbits were heparinized before the [illegible]

the muscle Platinum loops were firmly tied with silk thread to the tendon and the piece of the ventricular wall as closely as possible to the insertions of the muscle This arrangement left the papillary muscle fully intact.

The muscle was mounted vertically in the recording chamber (a jacketed thermostated Perspex bath with the lower end (the ventricular wall end) attached to a tension transducer (RCA 5734 and the upper end connected via a carefully straightened steel wire to a displacement transducer A detailed description of the recording device including the arrangements for damping of oscillations loading of muscle stimulation production of quick release and photographic recording of the signals from the transducers (1 tension 2 shortening 3 shortening velocity) have been given previously (Edman and Nilsson 1968) The equivalent mass of the displacement transducer used in this study was 86 mg and its static friction <2 mg The stray compliance of the recording device including the connections of the muscle with the transducers was 27 μ/g A preload of 100 mg was used throughout the experiments The preloaded muscle length i.e the distance between the insertions of the muscle to the tendon and the ventricle wall was determined by means of an ocular micrometer (10 × magnification) while the muscle was mounted in the bath. The muscle cross section was estimated on the basis of its wet weight and by assuming that the muscle had a cylindrical shape and a specific gravity of 1 0 The wet weight was determined at the end of the experiment after removal of the upper tendon and the portion of the ventricle wall to which the muscle had been attached

The muscle after being mounted to the recording device was paced to contract at 30 beats per minute for at least one hour before the experiment started The inotropic changes to be described were produced by altering the contraction frequency The effects refer to steady state conditions at each frequency studied

A Ringer's solution of the following composition was used (mM) NaCl 120 KCl 5 $NaHCO_3$ 25 NaH_2PO_4 1 5 $CaCl_2$ 2 0 $MgSO_4$ 1 5 Glucose 10 pH 7 40 The solution was continuously aerated with a mixture of 95 % O_2 + 5 % CO_2 during dissection of the muscle as well as during the actual experiment. Glass distilled water was used for washing of the glassware and for the preparation of the solutions All chemicals used were of analytical grade



Fig. 1. a. Schematic illustration of method for collection of active state data. Photographically superimposed oscilloscope traces illustrating isotonic shortening of rabbit papillary muscle after quick release at different times after the stimulus. The velocities of shortening are collected at the same degree of shortening of the muscle in each release record (at intersection of traces with dashed horizontal line). The velocity data considered as a function of time after the stimulus (dashed vertical lines) provide a measure of the course of the active state at a given length of the contractile element. b. Oscilloscope record illustrating isotonic shortening after quick release (upper) and electrically differentiated signal of the length transducer (lower).
Resting length 6.0 mm. Load 100 mg. 54 contractions per minute. Temp. 32° C.

Results

A. *The time course of the active state*

The active state was determined as the maximal speed of shortening of the contractile unit at different times after the stimulus using the same conceptual model and experimental approach as described in detail previously (Edman and Nilsson, 1968). *According to this technique the time course of the active state can be* analyzed at a precise length of the contractile unit, the individual active state data being collected when the muscle passes a given length. A few points of the experimental approach need to be reiterated. The muscle was paced to contract isometrically at a given frequency. During certain contractions, about 30 sec apart, the muscle was released at a selected time after the stimulus to shorten against a small load (100 mg). The length change took place in two steps: an initial rapid phase during which the series elastic element equilibrated with the load, and a subsequent less rapid phase representing the shortening of the contractile unit alone (Fig. 1). The velocity of shortening during the second phase at a given length of the muscle was used as an index of the active state in each case. The method of collecting the active state data is illustrated in Fig. 1a. Here a series of quick release records are superimposed on the same time base. The velocity of shortening is measured at the intersection of respective trace with the dashed horizontal line. The values so obtained considered as a function of time after the stimulus provide a measure of the course of the active state at a defined length of the contractile unit. The fact that the load was the same in all quick release recordings implies that the degree of stretch of the series elastic element was identical during the second shortening phase in each record. This means that a particular overall length of the muscle during the second

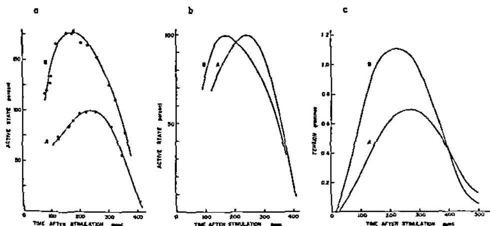


Fig 2 The time course of the active state (a and b) and the ordinary isometric myogram (c) of rabbit papillary muscle recorded at two different stimulation frequencies Curves A 30 contractions per min Curves B 72 contractions per min In left diagram (a) the intensity of the active state is expressed as percentage of the maximum recorded at 30 contractions per min , in the middle diagram (b) the maximum at both frequencies is normalized to 100 per cent Temp 29° C

phase represents a unique length state of the contractile unit [1] As was demonstrated previously for rabbit papillary muscle (Edman *et al* 1966, Edman and Nilsson 1968), the active state curve defined in terms of shortening velocity as just outlined, is probably also a valid index of the muscle's capacity to produce tension at the contractile element length considered

The experimental approach used here is similar to that devised by Jewell and Wilkie (1960) in studies on frog skeletal muscle and later adopted by Sonnenblick (1965, 1967) in experiments on cat papillary muscles These earlier versions of the technique, however, differed from the present one by not controlling for the intrinsic length changes of the muscle that occur during the (pseudo-)isometric contraction

The effects on the time course of the active state of the rabbit papillary muscle caused by a change in contraction frequency are illustrated in Fig 2 The curves at both frequencies are based on measurements at the same length of the contractile unit In Fig 2a the intensity of the active state is expressed as percentage of the maximal activity recorded at 30 contractions per minute, in Fig 2b the maximum of both curves has been normalized to 100 per cent As can be seen an increase in frequency from 30 to 72 per minute nearly doubled the intensity of the active state and produced a more rapid rise of the activity and an earlier attainment of maximal

[1] It should be noted that the term contractile unit refers to the functional model used It is an index of the ... idual active elements composing the preparation
The ... unit (in terms of the analytical model is kept
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tion ... the muscle has changed (see Gay and Johnson
1967

tained virtually constant. These results by extrapolation from the empirical data support the view that V_{max} and P_{max} are changed in parallel in response to the inotropic intervention studied.

Results divergent from those reported in this investigation have been described by Sonnenblick (1962, 1965) in studies of afterload contractions of cat papillary muscles. He found that the force velocity curve undergoes a non-congruent shift by altering the frequency of contraction of the muscle, leading to a greater change of V_{max} than of P_{max}. The reason for this discrepancy in the results is not clear. It should be noted, however, that the force velocity curve derived from measurements of afterload contractions does not represent a given state of activity of the muscle (for further discussion of this point see Blinks and Koch-Weser 1963, Edman 1965, Blinks and Jewell 1968, Brady 1968). The velocity data used for construction of the curve (the initial speed of shortening during the isotonic phase) refer to different times of the contraction period and to different length states of the contractile unit depending on the load studied. The complicated situation inherent in the afterload recording technique makes it difficult to evaluate quantitatively the change of the force-velocity relationship during an inotropic intervention of the myocardium.

C. *The series elastic element*

The stiffness of the series elastic element was determined as the drop in tension (ΔP) that occurred in response to a rapid controlled (about 1 per cent) release of the muscle (ΔL) at given tension levels (P) during isometric contraction. The details of the technical approach have been described previously (Edman and Nilsson 1968). Measurements of $\Delta P/\Delta L$ were performed both during the rising and falling phases of the contraction while the muscle was paced to contract at a given frequency. Eight experiments were performed in which the contraction frequency was varied between 18 and 72 contractions per minute.

The results are illustrated by two experiments in Fig. 4. It can be seen, in confirmation of previous results (Edman and Nilsson 1968, Parmley and Sonnenblick 1967), that the stiffness, $\Delta P/\Delta L$, at a given P, was the same irrespective of whether the measurement was made during the rising phase of the contraction or during relaxation. This implies, in view of the difference in the intensity of the active state between the contraction and relaxation phases (Fig. 2), that the series elastic element behaves as a purely passive entity: at least for tensions greater than 15 per cent of the peak twitch tension the properties of the series elastic component are independent of time and intensity of the active state.

Within the limits of experimental error the ratio $\Delta P/\Delta L$ to P was not affected by altering the contraction frequency notwithstanding the fact that the maximal intensity of the active state was greatly changed under the same conditions. The results would thus seem to make clear that the inotropic intervention produced by altered pacing of the cardiac muscle does not involve the passive elastic properties of the muscle to any significant degree. The inotropic effects shown in the ordinary isometric twitch myogram are therefore to be fully attributed to changes in the

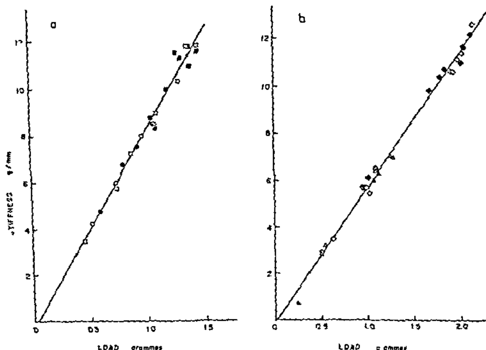


Fig 4 The stiffness of the series elastic element of two rabbit papillary muscles Measurements carried out at different tensions both during the rising phase (open symbols) and during the falling phase (filled symbols) of the isometric twitch a ● ○ 30 contractions per min ■ □ 60 contractions per min
b ▲ △ 18 contractions per min ◆ ◇ 36 contractions per min
Temp a 31° C b 23° C

functional state of the contractile unit as described in the preceding sections Lack of effect upon the series compliance of the myocardium has also been reported for inotropic interventions produced by digitalis calcium and adrenaline (Parmley and Sonnenblick 1967)

D *The isometric twitch*

The effects on the active state produced by altered pacing of the muscle described in section A were reflected in the isometric myogram (Fig 2) Thus in association with an increase in amplitude of the isometric twitch at the higher contraction frequency there was a reduction of the time to peak twitch tension and a decrease of the total contraction time The inotropic changes pictured by the isometric myogram are complicated however by the intrinsic length changes of the muscle which occur during contraction As was demonstrated in a previous study (Edman and Nilsson 1968) the course of the active state is not definitely programmed when the contraction starts the final outcome of the active state is critically dependent on the length assumed by the contractile unit from moment to moment during the

activity. For instance, by the amount of intrinsic shortening (5—8 per cent of the resting muscle length) normally taking place during an isometric contraction the peak intensity of the active state is decreased by approximately 45 per cent and the duration of the active state is reduced by 10—15 per cent (data refer to 30° C, 30 contractions per minute and 100—200 mg preload of the papillary muscle). Intensification of the active state as a result of an inotropic intervention of the muscle will cause a greater amount of shortening of the contractile unit during the activity period. This will mask the positive inotropic effect to a certain degree since the mechanical output is attenuated by reduction in length of the contractile element. Accordingly, the positive inotropic effect displayed by the isometric myogram is regularly found to be smaller than the change in intensity of the active state defined at a constant contractile element length.

In the experiment demonstrated in Fig. 2 the increase in peak intensity of the active state was 75 per cent as compared to 60 per cent increase in the isometric twitch amplitude. This difference can be fully accounted for by the different amounts of intrinsic shortening of the muscle during isometric contraction at the two inotropic states. As can be derived from load extension curves of the series elastic element (Fig. 8, Edman and Nilsson 1968), a 60 per cent increase in isometric twitch tension requires about 1.5 per cent additional stretch of the series elastic component with an equivalent amount of shortening of the contractile element. A decrease in length of the active unit by 1.5 per cent, however, will reduce the muscle's capacity to produce tension by a factor of 0.9 (Table I, Edman and Nilsson 1968). Thus, while the intensity of the active state (considered at a given length of the contractile unit) increases to 175 per cent of the control value, the isometric tension would be expected to rise to only 0.9×175 per cent, i.e. to 158 per cent, in good agreement with the actual findings in Fig. 2. It is clear from these results that the active state, rather than the ordinary isometric twitch response, is the proper parameter to use for a quantitative evaluation of an inotropic intervention. Only in this way is it possible to determine the inotropic effect without interference from length changes within the contractile system.

Discussion

Analysis of the dynamics of the mechanical activity of rabbit papillary muscles has shown that the concept of the active state, as originally formulated in the skeletal muscle (Hill 1949, also see Jewell and Wilkie 1960), is applicable to the contraction of the cardiac muscle (Edman and Nilsson 1968). It was found that the capacity of the heart muscle to develop tension (P_{max}) and the capacity to produce motion (V_{max}) undergo very nearly parallel changes during the course of a contraction. The mechanical behaviour of the muscle as displayed by the analysis of the active state may provide a relevant clue to the evaluation of the molecular events in the activation of the contractile element. It suggests that there exists a control mechanism in the excitation-contraction coupling which continuously regulates the mechanical out

put during the contraction in such a way that the ratio of V_{max} to P_{max} is kept constant all the time during the activity period The present results are fully consistent with this idea It was found (section B) that the inotropic effect produced by altered pacing of the muscle is a genuine change of the active state involving equally great changes of the muscle's capacity to produce tension and of its capacity to shorten actively It is essential to point out that the inotropic intervention does not merely affect the peak intensity of the active state, the course of the active state is fundamentally changed leading to an earlier onset of the activity and an earlier attainment of the maximum at the higher contraction frequency Taken together these findings support the assumption 1 that the two parameters of the myocardial contraction, force and shortening velocity, are governed by a common control mechanism in the excitation-contraction coupling and 2 that the inotropic effect is due to altered kinetics of this control function

The existence of a discrete activator step in the excitation contraction coupling, involving release of calcium or a calcium complex into the myofibrillar space, has been a favorite idea for several years (for references see recent review articles by Hasselbach 1964, Brady 1964, Huxley 1964 Klaus and Lullmann 1964, Edman 1965, Sandow 1965, Nayler 1966, Langer 1968) and attempts have been made to fit such a mechanism into the sliding filament model (Davies 1963) However, the theories of contraction advanced so far do not provide a ready explanation for the kinetics of the active state revealed by the present study of the myocardium According to the assumptions made in the original sliding filament hypothesis (Huxley 1957) and in a more recent version of this theory (Davies 1963) the activator governs the formation of active links between the A and I filaments but has no effect whatsoever on the dynamics of the active bridge The connection once it has been formed is assumed to run through a cycle of activity (thereby propelling the I filament over a given distance) at a speed determined by the load alone On this assumption P_{max} (the capacity to develop tension at zero velocity) would be quantitatively related to the number of active links and hence to the actual activator concentration V_{max} (the velocity of shortening at zero load) on the other hand being virtually independent of the number of active sites (Huxley 1957, Davies 1963), would be at its maximum as soon as there is activator enough to form some bridges The present results have clearly shown however that V_{max} does not change in an all or nothing fashion V_{max} and P_{max} both undergo gradual changes such that the ratio of V_{max} to P_{max} is constant during the course of a contraction and independent of the inotropic state Gradual changes of both V_{max} and P_{max} have also been demonstrated during twitch contraction of frog's skeletal muscle (Jewell and Wilkie 1960) and during calcium induced contraction of skinned amphibian muscle fibres (Endo 1967) These findings suggest contrary to previous assumptions, that the elementary process involved in the propulsion of the myofilaments can be adjusted in a graded way by means of changing the activator concentration in the intracellular medium

An alternative explanation of the gradual changes in V_{max} could be that V_{max} does not as assumed above represent the shortening velocity of the completely unloaded

contractile system It may be that the propulsion of the filaments is hampered by opposing frictional forces within the sarcomere, in which case V_{max} would correspond to a finite intrinsic load of the myofilaments Under such conditions then the estimated value for V_{max} would be expected to change in conjunction with a shift of P_{max}, while the real value for V_{max}, *i e* at zero intrinsic load, may be constant Results obtained in studies of skeletal muscle fibres (Gordon, Huxley and Julian 1966) would seem to indicate, however, that the resistance to shortening is small at sarcomere lengths greater than 1 65 μ, *i e* at the lengths concerned in the present investigation (see Gay and Johnson 1967) At any rate, even though some intrinsic load may exist, this would not account for the results quantitatively, *viz* the fact that V_{max} is affected by the same amount as P_{max} It would therefore seem that the changes in V_{max} observed during the course of a contraction, and in response to an inotropic intervention, do reflect changes in the functional state of the mechanism which is responsible for the propulsion of the myofilaments, as concluded above

If the active state is governed by a chemical activator it is logical to assume that the inotropic effects studied are due to altered kinetics of this agent in the myofibrillar space *The fact that the active state does not reach saturation during a contraction cycle*, under the conditions used means that every change in concentration of the activator around the myofibrils (above a certain threshold concentration) would manifest itself as a mechanical response The results have shown that while the intensity of the active state is increased there is a steeper initial rise and an earlier attainment of the maximum at the higher contraction frequency The duration of the decay phase of the active state on the other hand is not substantially changed (also see Gennser and Nilsson 1968) These observations considered together support the idea that the intensification of the active state does involve an increased rate of release or transport of the activator into the myofibrillar space as suggested previously in studies of the calcium metabolism of heart muscle (*e g* Winegrad and Shanes 1962 Niedergerke 1963 Klaus and Lullmann 1964 Nayler 1966 Fleckenstein 1968 Fuchs *et al* 1968 Langer 1968) On the same basis the reduced time to peak active state would seem to indicate that the duration of release of the activator is decreased at the higher contraction frequency

The authors wish to express their gratitude to Miss M Olsson for her able technical assistance during this investigation This work was supported by research grants from the Swedish Medical Research Council Project No 14X 184-04 and the Medical Faculty of the University of Lund

References

ABBOTT B C and W F H M MOMMAERTS A study of inotropic mechanisms in the papillary muscle preparation *J gen Physiol* 1959 *42* 533—551

BLINKS J R and J KOCH WESER Physical factors in the analysis of the actions of drugs on myocardial contractility *Pharmacol Rev* 1963 *15* 531 599

BLINKS J R and B R JEWELL Drugs and the mechanical properties of heart muscle *Ann Rev Pharmacol* 1968 *8* 113 130

BRADY A J Excitation and excitation-contraction coupling in cardiac muscle *Ann Rev Physiol* 1964 *26* 341—356

BRADY, A J, A measurement of the active state in papillary muscle *Physiologist* 1967 *10* 130

BRADY, A J, Active state in cardiac muscle *Physiol Rev* 1968 *48* 570—600

DAVIES, R E., A molecular theory of muscle contraction calcium-dependent contractions [illegible] he myosin actin [illegible] 1965 *5* 99—

EDMAN, K A P, D W GRIEVE and E NILSSON, Studies of the excitation-contraction mechanism in the skeletal muscle and the myocardium *Pflugers Arch ges Physiol* 1966 *290* 320—334

EDMAN, K A P and E NILSSON, The mechanical parameters of myocardial contraction studied at a constant length of the contractile element *Acta physiol scand* 1968 *72* 205—219

ENDO, M, Regulation of contraction relaxation cycle Proc 17th General Assembly *Jap Med Soc* 1967 *1* 193—197

FLECKENSTEIN A, *Elektromechanische Koppelung, in Heart Failure, Pathophysiological and clinical Aspects* (Eds Reindell, Keul and Doll) Georg Thieme Verlag, Stuttgart 1968

FUCHS, F, W GERTZ and F N BRIGGS, The effects of quinidine on calcium accumulation by sarcoplasmic reticulum of skeletal and cardiac muscle *J gen Physiol* 1968 *52* 955—968

GAY, W A and E A JOHNSON, An antomical evaluation of the myocardial length tension diagram *Circulat Res* 1967 *21* 33—43

GENNSER, G and E NILSSON, The relation between the action potential and the active state in human foetal myocardium and its dependence on muscle length and contraction frequency *Acta physiol scand* 1968 *73* 42—53

GORDON, A M, A F HUXLEY and F J JULIAN, The variation in isometric tension with sarcomere length in vertebrate muscle fibres *J Physiol* (Lond) 1966 *184* 170—192

HASSELBACH, W, Relaxing factor and the relaxation of muscle *Progr Biophys mol Biol* 1964 *14* 167—222

HILL A V The abrupt transition from rest to activity in muscle *Proc Roy Soc B* 1949 *136* 399—420

HUXLEY, A F, Muscle structure and theories of contraction *Progr Biophys* 1957 *7* 255—318

HUXLEY A F, Muscle *Ann Rev Physiol* 1964 *26* 131—152

JEWELL B R and D R WILKIE, The mechanical properties of relaxing muscle *J Physiol* (Lond) 1960 *152* 30—47

JOHNSON E A and R N SHEPHARD, Cardiac muscle under the microscope *Fed Proc* 1968 *27* 697

KLAUS, W and H LULLMANN, Calcium als intracellulare Übertragersubstanz und die mögliche Bedeutung dieser Mechanismus für die pharmakologische Wirkungen *Klin Wschr* 1964 *42* 253—259

[illegible] relation to myocardial contractility [illegible] ysiology of muscle contraction *Clin* [illegible] s of frog heart *J Physiol* (Lond)

PARMLEY W F and E H SONNENBLICK Series elasticity in heart muscle Its relation to contractile element velocity and proposed muscle models *Circulat Res* 1967 *20* 112—123

SANDOW A Excitation-contraction coupling in skeletal muscle *Pharmacol. Rev.* 1965, *17* 265—320

SONNENBLICK, E H Force-velocity relations in mammalian heart muscle *Amer J Physiol* 1962 *202* 931—939

SONNENBLICK E H Determinants of active state in heart muscle Force velocity, instantaneous muscle length time *Fed Proc* 1965 *24* 1396 1409

SONNENBLICK E H Active state in heart muscle Its delayed onset and modification by ionotropic agents *J gen Physiol* 1967 *50* 661—676

WINEGRAD A and A M SHANES Calcium flux and contractility in guinea pig atria *J gen Physiol* 1962 *45* 371—394

Acta physiol. scand. 1969. 76. 248—254

From the Department of Medicine, University Hospital, Lund, Sweden

On the Mechanism of Action for the Effect of Cholecystokinin on the Choledochoduodenal Junction in the Cat

By

PAVO HEDNER and GUNNAR RORSMAN

Received 13 September 1968

Abstract

HEDNER, P. and G. RORSMAN. *On the mechanism of action for the effect of cholecystokinin on the choledochoduodenal junction in the cat.* Acta physiol. scand. 1969. 76. 248—254.

The resistance of the choledochoduodenal junction was studied by measuring the flow from the common duct into the duodenum under constant infusion pressure and by measuring the intracholedochal pressure under constant infusion rate. It is concluded that the choledochoduodenal junction contains a real sphincter where the normal contracted state depends on an intact cholinergic nerve supply. As the effect of cholecystokinin on the small intestine is mediated by a cholinergic pathway, a similar mechanism of action for its effect on the choledochoduodenal junction was questioned. However, cholinergic and adrenergic mechanisms could be blocked by atropine and reserpine without affecting the antagonism of cholecystokinin to the contraction of the sphincter elicited by bradykinin. Thus the effect of cholecystokinin on the choledochoduodenal junction seems to be a direct one and related rather to its effect on the gall-bladder than to its effect on the small intestine.

Cholecystokinin has been shown to elicit a contraction of smooth muscle in two different organs by two different mechanisms of action. In the gall bladder the effect is not inhibited by atropine, indicating a possible effect directly on the smooth muscle cells. In the small intestine the stimulating effect of cholecystokinin is mediated by a cholinergic nervous pathway without cholinergic synapses (Hedner and Rorsman 1968). The gall bladder, the choledochoduodenal junction and the small intestine form a functional unit where cholecystokinin seems to be of physiological importance. The decreased resistance in the choledochoduodenal junction caused by cholecystokinin is a well known phenomenon, clearly demonstrated by Sandblom *et al.* (1935) and verified in numerous later investigations. The mechanism of action of this effect is, however, still unknown. With respect to the recent evidence for different mechanisms of action for the effect of cholecystokinin on the gall-bladder and on the small intestine respectively, it was considered to be of interest to investigate its effect on the choledochoduodenal junction to see if it could be related to either of these two organs.

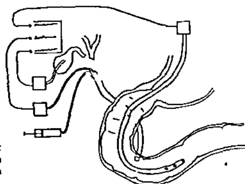


Fig 1 Schematic drawing of the arrangement of catheters and registration of pressures in experiments with a constant rate of infusion into the common bile duct

Material and methods

20 cats of either sex weighing 2 5—5 6 kg were used They were anaesthetized with pentobarbital which initially was given intraperitoneally and then the anaesthesia was maintained by intermittent i.v. infusion (0 1 mg/kg/hr) All animals were kept fasting for at least 15 hrs before operation Two different principles for registration of the choledochoduodenal resistance were used

A In 11 cats carotid blood pressure gall bladder pressure duodenal pressure at about 3 cm distal to papilla Vateri and the pressure in the common bile duct were simultaneously registered by fluid filled recording systems connected to a Grass polygraph recorder A soft polyethylene catheter was inserted into the gallbladder as shown in Fig 1 or via the cystic duct It was fixed by ligation and the proximal end of the common duct was also ligated In the distal part of the common duct two soft polyethylene catheters were inserted and fixed by ligation One of them was used for pressure recording and the other for continuous infusion of prewarmed Ringer s solution at a rate of 0 5 ml/min The intraduodenal pressure was measured by a polyethylene catheter passed through the oral cavity oesophagus and stomach (open tip) A schematic drawing of the arrangement of the catheters is presented in Fig 1

B Nine cats were prepared with carotid and duodenal catheters as described under A

ments

A preparation of cholecystokinin containing 1500 Ivy dog units per mg was used[1] It is referred to by the short form CCK in the following Doses given for 5 hydroxytryptamine refer to the creatinin sulphate and for atropine to the sulphate A synthetic preparation of bradykinin BRS 640 0 1 mg per ml was used[2]

Results

A *Constant infusion rate, intracholedochal pressure recording* When the infusion into the common duct began, there was after 10—20 sec a rapid increase of the intracholedochal pressure The records from the common duct showed as a rule a specific wave form profile and periodicity different from that in the duodenum and with pressure changes between 10 and 20 mm Hg (Fig 2)

When CCK was given (2 Ivy dog units per kg body weight intravenously) there was after a few seconds delay a fall in the intracholedochal pressure varying between

[1] Supplied by Prof E Jorpes Stockholm Sweden
[2] Supplied by Sandoz Produkter AB Täby Sweden

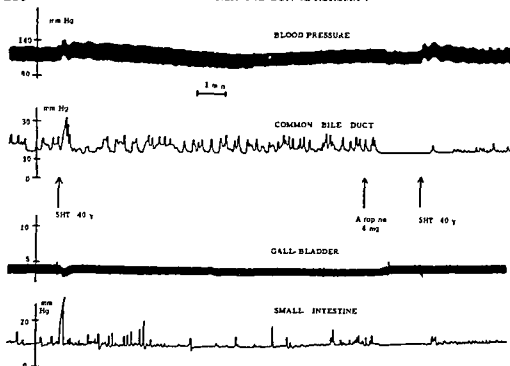


Fig 2 Wave formed pattern of the pressure in the common duct during infusion 0 5 ml per min Reduced effect of 5 hydroxytryptamine on the pressures in the common duct and in the duodenum after the i v administration of atropine

2 and 4 mm Hg (Fig 3) and the specific wave form pattern disappeared The pressure returned in 20—30 min to the preinjection level and the periodic pressure changes reappeared After CCK the pressure in the gall bladder increased with 2—5 mm Hg and in the duodenum numerous pressure peaks up to 30 mm Hg were registered

After atropine 1—2 mg per kg b w i v there was a fall of the intracholedochal pressure or disappearance of the phasic pressure changes a short and inconstant fall of the pressure in the gall bladder and disappearance of the spontaneous duodenal activity After atropine and also after CCK there was almost an equilibration between the intracholedochal and the intraduodenal pressures (Fig 2 Fig 3) After the administration of atropine the effect of CCK on the gall bladder was unaffected the pressure peaks in the duodenum otherwise observed were inhibited and there was no additional decrease of the pressure in the common duct

5 hydroxytryptamine 10 μg per kg i v elicited a peakformed rise of both intracholedochal and intraduodenal pressures amounting to 5—20 mm Hg and 10—20 mm Hg respectively At the same time there was generally a slight pressure fall in the gall bladder After pretreatment with atropine there was a marked reduction of the pressure peaks following 5 hydroxytryptamine (Fig 2)

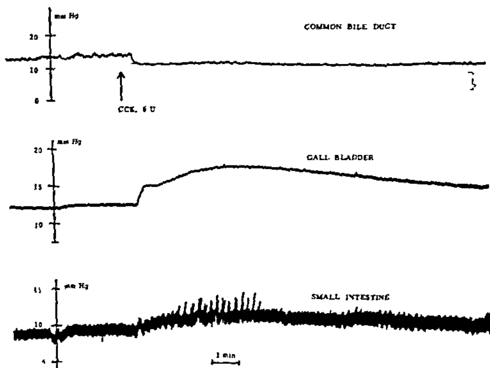


Fig 3 Effect of CCK on the pressure in the small intestine gallbladder and common bile duct

B. *Constant infusion pressure flow rate recording* For registration of both increased and decreased flow rates an infusion rate of 3—5 drops/min was found to be suitable It was usually produced by an infusion pressure of about 20 cm H_2O With lower pressures the flow rate decreased When the infusion pressure came close to the intraduodenal pressure the flow stopped completely also after atropine or CCK which otherwise considerably increased the flow rate

In some cases a group-wise arrangement of the drop markings was observed in the control period before any drug had been administered

In all experiments the administration of atropine (1—2 mg per kg) or CCK (2 units per kg) increased the flow rate considerably (Fig 4) For investigation of the effect of CCK in the atropinized animal we searched for a substance able to reduce the flow unaffected by atropine Morphine (2 mg per kg) was effective in reducing the flow rate before atropine but after atropinization the effect was inconstant and unreliable Histamine reduced the flow but the duration of its effect was inconstant which seemed to depend on a rapidly developing tachyphylaxis However bradykinin was found to meet the requirements a dose of 10 µg per kg usually producing a complete arrest of the flow through the catheter in the common duct for 10—15 min After atropine (1—2 mg per kg) the effect remained the same but ceased earlier which was easily compensated by increasing the dose with *e.g.* 50 % (see

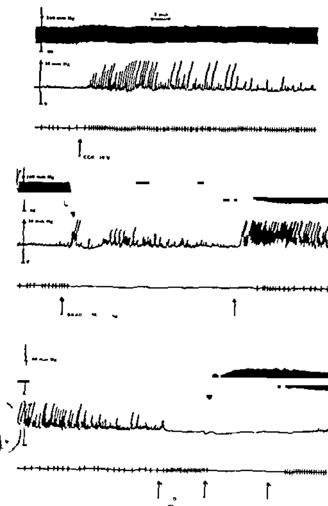


Fig 4 Effect of CCK atropine and bradykinin on blood pressure (upper record) duodenal pressure (middle record) and flow rate through the choledochoduodenal junction at a constant infusion pressure of 20 cm H_2O (lower record) Each marking on the lower record indicates one drop passing through the drop counter

Fig 4) Close to the injection of bradykinin a fall in blood pressure was registered also after pretreatment with atropine and a short lasting increase of the intraduodenal pressure was noted which was reduced or absent after atropine The flow stopped after bradykinin but started again about 1 min after the administration of CCK This effect remained the same also after pretreatment with atropine in all experiments (Fig 4) The same result was obtained also in all animals that had been pretreated with reserpine

Discussion

The two principles of registration used here have both been used in several investigations reported in the literature and both have been regarded to give representative recordings of the resistance in the choledochoduodenal junction This is supported by the fact that we found them to agree under comparable experimental conditions

Following an increased intracholedochal pressure Bergh and Layne (1940) found a phasic reduction of the flow from the common duct to the duodenum which was not related to the duodenal activity Hauge and Mark (1965) reported an independent intrinsic motor activity of the terminal part of the common duct After atropine we found almost an equilibration of the pressures in the common duct and in the duodenum. These findings together with anatomical evidence (Boyden 1957) imply that the choledochoduodenal junction contains a real sphincter able to operate independently of the duodenal wall In this sphincter the contraction seems to be the active process and a dilatation is achieved by relaxation Under physiological conditions the contraction seems to be dependent on an intact cholinergic nerve supply

Watts and Dunphy (1966) have suggested that the sphincter unit may play a more active role in the transport of bile by actively pumping bile into the duodenum an activity that might be stimulated by cholecystokinin This has been denied by others (Wyatt 1968) Our experiments do not permit any definite conclusion in this question but we did not find any evidence for a transport of bile over the choledochoduodenal junction against a pressure gradient

An active dilatator in the choledochoduodenal junction stimulated by cholecystokinin remains as a possibility but hitherto no anatomic or physiologic evidence for such a mechanism has to our knowledge been presented

Like several other investigators we found in some experiments a change between a higher and a lower pressure in the common duct during the infusion with a periodicity different from that in the duodenum This may imply an intrinsic activity of the sphincter unit as suggested by Sitnick *et al* (1964) who observed a phasic pressure pattern in the common duct in the dog It may also be explained by a reflex mechanism by which an elevated pressure in the common duct might cause the sphincter unit to emit bile Such a reflex is still hypothetic but may deserve consideration as Wyatt (1967) found an increased flow through the choledochoduodenal junction following electric or mechanic stimuli applied to the gall bladder as a reflex relayed over the coeliac ganglion

As cholecystokinin has been shown to stimulate the small intestine via a cholinergic nervous pathway (Hedner and Rorsman 1968) the nervous supply of the choledochoduodenal junction might provide a similar mechanism of action for CCK on this organ When the normal contracted state of the sphincter had been inhibited by atropine no further effect on the pressure in the common duct was obtained by CCK Bradykinin which caused only a transient increase of the intraduodenal pressure now elicited a new contraction probably via a direct effect on the smooth muscle in the sphincter This contraction was antagonized by CCK also after the administration of atropine Thus the effect of CCK does not seem to depend on an intact cholinergic pathway As the same result was obtained in all animals pretreated with reserpine in a dose known to rule out the histologic appearance of adrenergic structures Carlsson 1966 an adrenergic mechanism does not seem to be involved As 5 hydroxytryptamine increased the intracholedochal

pressure it is reasonable to exclude a liberation of 5 hydroxytryptamine as the mechanism of action for CCK, which had to be considered when the effect of CCK on the small intestine was investigated (Hedner and Rorsman 1968)

Irrespective of the intricate arrangement of the choledochoduodenal junction the increased flow through it following CCK seems to be independent of intact cholinergic or adrenergic pathways a mechanism of action related rather to the gall bladder than to the small intestine.

We are indebted to Draco AB Lund for kind permission to use laboratories and technical equipment.

We wish to thank Mrs Margit Prytz for excellent technical assistance

References

Bergh G S and J A Layne A demonstration of the independent contraction of the sphincter of the common bile duct in human subjects. *Amer J Physiol* 1940 *128* 690—694

Boyden E A The choledochoduodenal junction in the cat. *Surgery* 1937 *41* 773—786

Carlsson A. Drugs which block the storage of 5 hydroxytryptamine and related amines In *Handbuch der experimentellen Pharmakologie* Editor W Erspamer 1966 *19* 529—592.

Hauge C W and J B D Mark Common bile duct motility and spincter mechanism I Pressure measurements with multiple lumen catheter in dogs *Ann Surg* 1965 *162* 1028—1038

Hedner, P and G Rorsman Structures essential for the effect of cholecystokinin on the guinea pig small intestine in vitro *Acta physiol scand* 1968 *74* 58—68

Sandblom Ph W L Voegtlin and A C Ivy The effect of cholecystokinin on the choledochoduodenal mechanism sphincter of Oddi) *Amer J Physiol* 1935 *113* 175—180

…tnick J L W D Warren and M B Klein The evaluation of spincter of Oddi function. *Surg Forum* 1964 *15* 373—375

Watts J M and J E Dunphy The role of the common bile duct in biliary dynamics *Surg Gynec Obstet* 1966 *122* 1207—1218

Wyatt A P The relationship of the spincter of Oddi to the stomach duodenum and gall bladder *J Physiol* Lond 1967 *193* 225—243

Wyatt A P Dynamics of the common duct *Lancet* 1968 *I* 310

Acta physiol. scand. 1969. 76. 255—256

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Acute Neuromuscular Transmission Failure in Vas Deferens after Reserpine

By

U. S. von Euler

During a study of the acute effects of reserpine on isolated organs it was observed that this drug in low concentrations caused a block of the neuromuscular transmission in the guinea pig vas deferens.

Vas deferens was prepared according to Hukovic (1961) and mounted in a 20 ml bath with Tyrode solution at 37° C. The hypogastric nerve was stimulated by a Grass Stimulator for 5 sec at 1 min intervals, 3 msec duration and a frequency of 20—60 stimuli/sec. Voltage was adjusted to give maximal response. Contractions were recorded semi-isometrically by a potentiometer recorder with a Grass Force Displacement Transducer system.

After stabilization of the stimulatory response, reserpine (Serpasil® CIBA) was added to the bath in concentrations of 0.05—0.5 μg/ml bath fluid and the contractions recorded (Fig. 1). As seen in the figure the contractions subsided rapidly after reserpine in the highest concentration and were reduced to a barely perceptible response. At the lowest concentration of reserpine tested, 0.05 μg/ml, the blocking effect was weak but manifested itself clearly although relatively slowly on further addition of 0.1 μg/ml reserpine. Serpasil solvent in corresponding amounts had no effect on the contractions. The blocking effect could be readily abolished by washing with fresh Tyrode and 10—20 min rest.

In other experiments the isolated organ was subjected to postganglionic nerve stimulation according to Birmingham and Wilson (1963), with similar results indicating an effect of reserpine on the neuromuscular transmission.

An acute blocking effect of reserpine on the vasoconstrictor effect obtained by stimulation of the sympathetic nerves to the rabbit ear has previously been reported by Day and Owen (1968). The authors did not, however, comment on this finding.

The effect observed cannot be ascribed to an overall depletion of the transmitter content of the organ. Moreover, the stimulus response returned after washing and rest and was well maintained for at least 1 hr after the block. It seems more likely that the block is functional and depending on local deficience of transmitter immediately available for release.

Reserpine inhibits the transmitter release from isolated nerve granules (Euler and Lishajko 1963). This is also the case for granules prepared from accessory male genital organs which normally appear to have a slower release rate than splenic nerves or heart granules (Euler 1966 a).

Fig 1 Guinea pig vas deferens suspended in Tyrode solution Contractions elicited by electrical stimulation of the hypogastric nerve 5 sec at 1 min intervals Serpasil solvent 1 4000, Serpasil same volume (0 5 µg/ml) 0 05 µg/ml reserpine (Serpasil®), 0 1 µg/ml reserpine At ×6 min interval

It has been assumed that nervous stimuli release the transmitter from sites either in the terminal axon membrane itself or from closely associated axonal sites and further that a dynamic equilibrium exists between these and the granular stores (Euler 1966 b) A lowered release rate from the granules, as shown to occur after reserpine may therefore cause a gradual decrease in the amount of transmitter transferred to the actual neuronal release sites In agreement with this concept the first stimulus response after addition of reserpine is normal, whereafter the contractions decline gradually at a rate depending on the dose After removal of the blocking agent and after rest the equilibrium may be re-established and the normal response reappears A similar type of transmitter deficiency, but due to inhibition of resynthesis, has been described after decaborane on stimulation of the lumbar sympathetic in the rabbit (Bygdeman and Euler 1966)

It is suggested that the hypotensive effect of small doses of reserpine in man may be due to retarded release rather than to depletion of the main stores

The research reported has been sponsored in part by the Swedish Medical Research Council under project no B69-14X 97 05A and Knut and Alice Wallenberg Foundation The gift of Serpasil by CIBA Produkter, Vallingby, is gratefully acknowledged Thanks are due to mrs Anita Jorgensen for skilful assistance

References

BIRMINGHAM A T and A B WILSON Preganglionic and postganglionic stimulation of the guinea pig isolated vas deferens preparation *Brit J Pharmacol* 1963 *21* 569—580

BYGDEMAN S and U S v EULER Neurotransmitter deficiency and reloading in noradrenaline depleted rabbits *Acta physiol scand* 1966 *68* 134—140

DAY, M D and D A A OWEN The interaction between angiotensin and sympathetic vasoconstriction in the isolated artery of the rabbit ear *Brit J Pharmacol* 1968 *34* 499—507

EULER U S v Release and uptake of noradrenaline in adrenergic nerve granules *Acta physiol scand* 1966 a *67* 430—440

EULER U S v 20 Years Noradrenaline *Pharmacol Rev* 1966 b *18* 29—38

EULER U S v and F LISHAJKO Effect of reserpine on the uptake of catecholamines in isolated nerve storage granules *Int J Neuropharmacol* 1963 *2* 127—134

HUKOVIC, S Responses of the isolated sympathetic nerve ductus deference preparation of the guinea pig *Brit J Pharmacol* 1961 *16* 188—194

Abstracts from Meeting of the Scandinavian Physiological Society in København 11—12 April 1969.

DEMONSTRATIONS

D 1

Institute of Medical Physiology, Department A, University of Copenhagen, Denmark

Recording of Cell Membrane Potentials in Perfused Rat Liver

By

M. Folke

D 2

Institute of Medical Physiology B, University of Copenhagen, Denmark

Electrical and Mechanical Activity in Myometrial Grafts in the Rabbit

By

G. Wagner

By placing a homograft of myometrial tissue ($1 \times 1 \times 1$ mm) into a previously inserted ear-chamber it has been possible to obtain electrical signals via platinum electrodes one of which protruded into the graft. Contractions of the tissue—either induced by oxytocin or arised spontaneously—result in blanching of the graft. This can be recorded by means of the change in the transillumination. Long term studies of the spontaneous activity and response to oxytocin during pregnancy as well as during and after spontaneous delivery demonstrate that the graft follows the same pattern as that described for the organ in situ.

The long term studies were made possible by the use of a—for the purpose constructed—FM telemetry transmitter with a performance period of 30 days. The insulated wires from the electrodes were connected to the input of the transmitter. In this way the animal can be in its normal surroundings and move freely, as the transmitter can be implanted.

Fig. 1 demonstrates diagrammatically the transmission and the recording system.

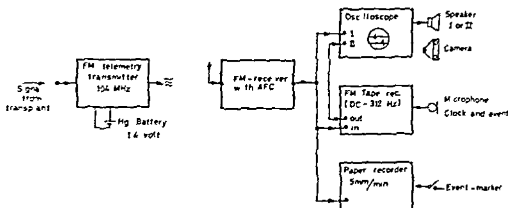


Fig 1 Gorm Wagner Transmission system and recording system.

As sympathectomy was performed in the neck before the grafting to the ear neither neural nor mechanical factors seem to be necessary for the normal function of the myometrium.

The method may be of use in pharmacological testing as well as in physiological studies

Records have been obtained during normal delivery and a series of contractions in the graft concomitant with the delivery of the fetuses and placentae occurred

Method and results will be demonstrated

D 3

Laboratoriet for klinisk arbejdsfysiologi, Rigshospitalet København Ø, Denmark

Krogh's Bicycle Ergometer Modified for Eccentric Exercise

By

F B Petersen

If a contracted muscle is stretched the mechanical tension will increase without any alterations in electromyographical activity, *i e* the number of active muscle fibres is the same. This form of muscle activity is called eccentric contraction and the resulting external output is generally called negative or eccentric work or exercise In fact it is not a work which is done but a braking force which is exerted upon the surroundings. During eccentric exercise it is possible to increase the tension in muscles, without increasing the energy consumed by the muscle It is, therefore, of interest to study the effect of eccentric exercise upon cardiac output, periferal circulation, body temperature and respiration This could give valuable information about the regulation of these parameters during muscular work Asmussen (1952)

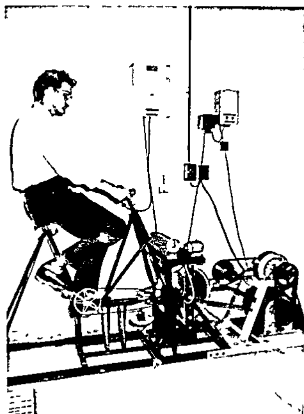


Fig 1 The modified Krogh's bicycle ergometer showing the electromotor, the induction clutch and on the wall the power supply

and Muller (1953) investigated the influence of eccentric exercise upon oxygen consumption Asmussen used a special bicycle and a tread mill with different inclinations and speeds However, the experiments called for considerable skill on the part of the experimental subjects, and a certain period of training due to the difficulty in balancing the bicycle on the tread mill It was thought of interest therefore, to develop a bicycle ergometer for negative work

A stepless variable induction clutch is combined with a Krogh's bicycle ergometer In this way it is possible to transmit known work loads from an electromotor to the Krogh ergometer The work load can be calibrated by the magnetic brake of the Krogh ergometer and when the direction of revolution is reversed it is possible for the subject to brake the ergometer by either the arms or the legs It is concluded that the device may be useful in investigating the eccentric dynamic work with arms and legs

References

ASMUSSEN E *Acta physiol scand 28* 1952 364 388
MÜLLER, E A, *Arbeitsphysiologie* 1953 *15* 196 200

C 1

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What Drives Water Across Epithelial Cells in the Absence of a General Transmural Osmotic Gradient?

By

E SKAUDHAUGE

C 2

Institute of Neurophysiology, University of Oslo Norway

Lamellar Organization of Hippocampal Excitatory Pathways

By

P ANDERSEN T V P BLISS, T LOMO, L I OLSEN and K K. SKREDE

We have investigated the distribution of excitatory impulses from the entorhinal area to the dentate area and the hippocampal fields CA3 and CA1 along the four membered pathway shown in Fig 1D

The hippocampal formation was stimulated with tungsten microelectrodes and recordings made with glass microelectrodes In Fig 1B the open and filled circles give the size of the antidromic CA3 population spike recorded from two sites (Rec 1 and Rec 2) in response to stimulation of Schaffer collaterals at a depth of 1 0 mm along the tracks *a* to *k* (Fig 1A) Similarly the open and filled triangles represent orthodromic CA3 responses to stimulation of dentate granule cells deeper in the same tracks (1 5 mm) The graph shows that fibres of both paths course along a strip as indicated by the dotted areas of Fig 1A Fig 1C shows some of the responses on which the graph is based The middle column gives the responses to on beam stimulation. The two columns on either side show the attenuation produced by moving the stimulating electrode 1 mm medially or laterally A similar organization was also found for the perforant and alvear paths (Fig 1C pp and alv) which represent the input and output fibres of the hippocampal formation, respectively

We conclude that axons of the four membered pathway originating from a group of entorhinal cells run in the same near sagittal plane from the point of entry at the hippocampal border via the dentate area and CA3 to CA1 As a result of this lamellar

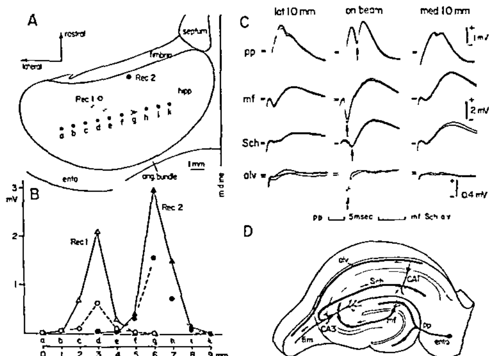


Fig 1 A Dorsolateral view of the hippocampal surface Rec 1 and Rec 2 entry points of CA3 recording electrodes *a—k* stimulating electrode tracks for orthodromic and antidromic CA3 activation B Amplitude of antidromic (circles) and orthodromic (triangles) population spikes evoked at Rec 1 and Rec 2 by stimulation at points *a—k* C Population spikes (arrows) evoked in the dentate area (pp), CA3 (Sch and mf) and CA1 (alv) by stimulation on beam (centre column) and 1 mm to either side (left and right columns) alv alveus mf mossy fibres. pp perforant path Sch Schaffer collaterals D Diagram of the proposed lamellar organization, with the four membered pathway heavily outlined

organization, local activation of the entorhinal area produces a beam of sequentially activated neurones confined to a narrow strip of the hippocampus as diagrammatically illustrated in Fig 1D

C 3

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Projections of Low Threshold Joint Afferents to the Cerebral Cortex of the Cat

By

L Korner and S Landgren

Low threshold joint afferents are considered to subserve kinesthesis (Rose and Mountcastle 1959) It is therefore important to study their projections to the cerebral

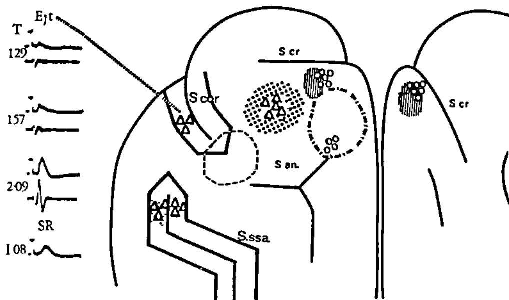


Fig 1 Records of potentials evoked by electrical stimulation of the contralateral elbow joint nerve and diagram showing the projection areas of the low threshold joint afferents in the somatosensory cortex

Upper records Cortical potentials evoked in the coronal sulcus Average of 50 sweeps Calibration pulse 5 msec 100 μV
Lower records Afferent volley in the musculocutaneous nerve
Δ Projections of low threshold elbow joint afferents
○ Projections of low threshold knee joint afferents
Stippling Projections of group I muscle afferents from the forelimb
Hatching Projections of Group I muscle afferents from the hindlimb
Broken line Caudal projection area of the superficial radial nerve
Dash dot line Dorsal projection area of the sural nerves
Scr Sulcus cruciatus
Scor Sulcus coronalis
San Sulcus ansatus
Sssa Sulcus suprasylvius anterior
Lines on both sides of Scor and Sssa indicate the two banks of these sulci

cortex The present knowledge of these projections is unsatisfactory There is disagreement about the existence of projections from the most low threshold group, and cortical responses of short latency indicating primary projections were so far not observed (*cf* Andersen *et al* 1967)

Cortical potentials evoked by graded electrical stimulation of low threshold afferents (Golgi and Ruffini endings) in the contralateral musculocutaneous elbow joint branch and the posterior knee joint nerve were recorded in cats anesthetized with chloralose The afferent volley was monitored Cortical potentials were recorded from the surface and from layers 3—5 A Didac computor was used to disclose responses of low amplitude

Low threshold afferents from the elbow joint project to the postcruciate dimple (Pcd) and to the anterior suprasylvian sulcus (Sssa) in both cases overlapping the projections of the low threshold muscle afferents (Fig 1) In addition there was a

projection area in the coronal sulcus near the caudal projections of the superficial radial nerve In the coronal area the response appeared with the first sign of the afferent volley, and the latency was similar to that of the cutaneous primary projections (4 5 msec) The latencies in Pcd and Sssa was 1—1 5 msec longer

Projections from the low threshold knee joint afferents overlapped with the dorsal and medial areas of the low threshold muscle afferents in the postsigmoid gyrus (*cf* Landgren and Silfvenius 1968) A third area was observed just rostral to the medial end of the ansate sulcus overlapping with the sural skin afferent projections

The ascending paths of the low threshold joint afferents were examined by spinal cord lesions at C_4 The elbow joint potentials were abolished by transection of the dorsal columns which did not affect the knee joint potentials These were abolished by transectioning of the dorsolateral fascicle The organization of the ascending paths is therefore similar to that of the low threshold muscle afferents

References

ANDERSEN H T L KORNER S LANDGREN and H SILFVENIUS *Acta physiol scand* 1967 *69* 373—382

LANDGREN S and H SILFVENIUS *Acta physiol scand* 1968 *73* 14A—15A.

ROSE J E and V B MOUNTCASTLE *Handbook of Physiology* Ed J Field William and Wilkins Co Baltimore 1959 *1* 387—429

C 4

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A Comparison of Two Methods of Assessing Capillary Permeability in Isolated Perfused Muscle Based on Single Injection Technique

By

C CRONE and D GARLICK

The application of indicator diffusion technique in the study of capillary permeability rests on the assumption of the existence of an extracted fraction E *i e* the portion of intraarterially injected diffusible material which has left the capillaries to reach the interstitium This fraction can be measured by using a non diffusible reference tracer together with the diffusible tracer The expression for capillary permeability in terms of extraction is $P = -(Q/A) \times \ln(1-E)$ (Crone 1963) where Q is blood flow and A is capillary surface area

In view of the importance of the measurement of E an independent estimate of this parameter would be valuable According to Johnson and Wilson (1966) it should be possible to determine E during the washout period after a bolus injection when the material which has diffused out of the capillaries returns again Washout extraction $E = kV/Q$ where k is the rate constant of the exponential clearance curve, V is the interstitial volume of distribution and Q the blood flow

Isolated gastrocnemius muscles of cats were perfused in a non recirculating system

with Ringer-colloid fluid at constant high flow (20—50 ml · min⁻¹ · 100 g⁻¹) The non-diffusible tracer was albumin-bound T-1824 The diffusible tracer was EDTA-Cr^{51} (MW 390), on extracellularly distributed γ-emitter From the time of bolus injection the external counting rate over the muscle was recorded with a ratemeter and printout Also commencing from injection time venous samples were collected for up to 60 min and aliquots measured for dye concentration and radioactivity Vascular and interstitial volumes were calculated from the venous concentration versus time curves of the reference and diffusible tracers respectively using the stochastic formulae of Zierler (1963)

In all experiments the extraction calculated from the washout period was less than half the extraction calculated by indicator dilution.

The use of a rate constant in the calculation of the extraction in the washout period assumes that no interstitial concentration gradients occur i.e. that the interstitial fluid is well mixed with respect to the tracer molecules

The presence of unstirred layers in the interstitium of muscle may, however, lead to diffusion gradients and thus explain the apparent discrepancy between the two methods of determining extraction.

References

Crone, C. *Acta physiol. scand* 1963 58 292—305
Johnson J A and T A Wilson *Amer J Physiol.* 1966 210 1299—1303
Zierler, K. *Circulat Res.* 1963 12 464—469

C 5

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The Capillary Permeability of Oxygen, Ions and Some Organic Compounds

By

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C 6

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The Disappearance of Xe^{133} and I^{125} in the Canine Gastrocnemius Muscle Following Vasoconstriction and Vasodilatation

By

P Bolme and L. Edwall

The aim of the study was to compare changes in the blood flow with changes in disappearance rate of a lipid soluble (Xe^{133}) and a water soluble (I^{125}) tracer during vasoconstriction and during different types of vasodilatation, the principal reason

being that in a recent study the changes in disappearance rate sometimes appreciably deviated from the recorded blood flow changes (Bolme and Edwall 1968)

The disappearance of Xe^{133} and I^{125} was followed in the isolated gastrocnemius muscle of anesthetized dogs by an external detector The blood flow was measured by an arterial electromagnetic flowmeter probe or by a venous drop recorder Graded vasoconstrictions were produced by electrical stimulation of the cut lumbar sympathetic trunk Vasodilatations were instituted by stimulation of the mixed tibial nerve producing metabolic vasodilatation or by i a infusion of acetylcholine The responses were expressed as the ratio between percentage change in disappearance rate and percentage change in the blood flow, (disappearance flow ratio)

During vasoconstriction blood flow and disappearance rate were decreased to the same extent resulting in a disappearance flow ratio of approximately 1 regardless of whether Xe^{133} or I^{125} were used

During metabolic vasodilatation the disappearance flow ratio usually exceeded 1 (range 0 3—11) This was observed during the whole period of vasodilatation In some experiments the blood flow was kept constant at resting level during motor nerve stimulation An increase in the disappearance rate was then regularly observed The results were about the same whether Xe^{133} or I^{125} were used

The effects of acetylcholine differed in some respects from those observed during metabolic vasodilatation Thus the initial effects on disappearance flow ratio were less Furthermore in late phases of acetylcholine infusion the disappearance rate approached control levels in spite of a continued high blood flow The observed differences between metabolic and acetylcholine induced vasodilatations were most prominent with I^{125}

The differences observed between the two types of vasodilatation were probably due to different effects exerted by them on the exchange surface area The results support the view that the disappearance of hydrophilic tracers is dependent on the available surface area The results also indicate that even for lipophilic solutes the disappearance is influenced by changes in the available surface area during vasodilatation

References

BOLME P and L EDWALL *Acta physiol scand* In press

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Capillary Diffusion Capacity for Small Hydrophilic Molecules in the Exercising Forearm of Man

By

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This investigation was undertaken in order to measure the capillary permeability for small hydrophilic substances in the exercising muscle of man

The extraction fraction, E for $^{24}Na^+$, $^{131}I^-$, and ^{51}Cr-EDTA was measured by the indicator diffusion method in exercising human forearm in twentythree normal subjects. The fractional loss of these diffusible indicators relative to the non-diffusible tracer (T-1824) was practically constant during the steeply rising part of the time-concentration curve. This finding is in agreement with the predictions that can be made from the "single capillary" model of Crone (1963), which postulates that the interstitial fluid space functions as a sink and that the unidirectional loss of diffusible tracer (the extraction fraction, E) *can* be assessed. E calculated as the ratio between the areas under the steeply raising part of the time concentration curves for the non-diffusible (T-1824) and diffusible tracer was $E_{Na}=0.55\pm0.07$, $E_I=0.40\pm0.05$ and $E_{Cr-EDTA}=0.23\pm0.05$. The forearm blood flow, Q calculated from the T 1824 time concentration curves was in the range of 22.0—50.0 ml whole blood per 100 g muscle per minute. From these values the muscle capillary diffusion capacity, CDC defined as the maximal unidirectional flux of diffusible tracer over the capillary membrane per unit concentration difference, was obtained in the unit of mole per (mole/ml) per 100 g muscle per minute, yielding CDC_{Na} 15.7±1.0, $CDC_I=13.1\pm1.8$ and $CDC_{Cr-EDTA}=3.67\pm0.49$ mole · (mole/ml)$^{-1}$ · 100 g^{-1} min^{-1}.

The ratio of the CDC for the two monovalent anions agreed with the corresponding ratio of their free diffusion coefficient in water. This supports the concept that diffusion restriction does appreciably not influence so small hydrophilic molecules in the muscle capillary. However restriction for diffusion apparently sets in at a given molecular size since proteins do not penetrate the small pore system available for small hydrophilic molecules. Preliminary studies of the inulin/sucrose CDC ratio ...nts to a larger value (in the range of 5—10) than that of the corresponding free diffusion coefficents (c. 3.5). This is in agreement with Pappenheimer's (1953) prediction of a pore diameter in the order of magnitude of 100 Å (*cf.* also the size of the intracellular gaps seen electromicroscopically between the endothelial cells). The ratios of the CDC for the cation to any one of the anions were somewhat larger than the corresponding ratio for the free diffusion coefficients suggesting that the anions transcapillary passage is impeded by electric charges at the level of the capillary membrane.

References

CRONE, C. *Acta physiol. scand.* 1963. 58. 292—305.
PAPPENHEIMER, J. R. *Physiol. Rev.* 1953. 33. 387—423.

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Non-Homogeneous Wash-Out Conditions of Xenon-133 from Adipose Tissue

By

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The wash-out of the radioactive inert gases from adipose tissue has been supposed to

take place with constant clearance rate (Larsen *et al* 1966) The clearance rate (k) has been used for calculation of the adipose tissue blood flow (f) by means of the blood to tissue partition coefficient (λ) for Xenon 133 $f = k \cdot \lambda \cdot$ 100 ml/min 100 g The purpose of the present study was to investigate whether the clearance rate of Xenon 133 from adipose tissue is constant and whether there is correlation between the calculated blood flow and the directly measured flow

The inguinal subcutaneous adipose tissue in urethane anesthetized rabbits was isolated with intact blood supply through the superficial epigastric vessels and then covered by a gas tight polyethylene membrane The venous effluent was continuously measured by a drop recorder unit and the blood was returned to the animal through an open reservoir The blood pressure blood flow and hematocrit were kept constant as well as the temperature The blood to tissue partition coefficient for Xenon was determined from the solubility in the blood and tissue components Xenon 133 activity was registered by scintillation crystals

Three types of experiments were performed

1 After *local atraumatic labeling* of the tissue by diffusion of Xenon 133 gas from the surface the externally registered wash out curves were strictly monoexponential and the calculated blood flow was in good agreement with the directly measured flow ($p < 0.01$)

2 After a *bolus injection* (0 1 ml saline containing Xenon 133 intra arterially in 3 sec) the externally registered wash out curves showed a faster clearance rate during the first 60—90 min but the blood flow calculated from the height area relation according to Zierler (1965) was in good agreement with the directly measured flow

3 When the wash out curves after bolus injection were registered both externally and over the venous effluent the initially faster clearance rate was much more pronounced on the venous side

It is concluded from the results that calculation of the adipose tissue blood flow from the externally registered Xenon 133 wash out curves from saturated tissue gives correct values for the blood flow This means that the clearance is flow limited However the initially faster clearance rate after bolus injection has shown that the wash out conditions for Xenon 133 from adipose tissue are not homogeneous Shunting by diffusion might be the explanation of this finding as it has been described for muscle tissue (Sejrsen and Tønnesen 1968) although the vascular structure in adipose tissue should favour complete mixing within the system

References

LARSEN O A N A LASSEN and F QUAADE *Acta physiol scand* 1966 *66* 337—345
SEJRSEN P and K H TONNESEN *Circulat Res* 1968 *22* 679—693
ZIERLER K *Circulat Res* 1965 *16* 309—321

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Brown Fat Thermogenesis Reflected in Mitochondrial Respiration

By

H. T. ANDERSEN, E. N. CHRISTIANSEN, H. J. GRAV and J. I. PEDERSEN

Brown adipose tissue is a major source of non-shivering thermogenesis. Its great thermogenic capacity is supposedly called upon in the neo-natal state, during arousal from hibernation and in cold adaptation.

It has been shown previously in this laboratory (Pedersen *et al.* 1968) that brown fat mitochondria from new-born guinea pigs exhibit a very low degree of respiration-linked phosphorylation. Thus when pyruvate-malate was used as substrate a P/O ratio of 0.47 ± 0.16 was observed whereas brain mitochondria from the same animals yielded a P/O ratio ranging from 2.56 to 1.97 which is fairly close to the theoretical value of 3. At age 3 weeks, however, the P/O ratio of the brown fat mitochondria had increased to 2.60 ± 0.61. These results indicate that the great thermogenic capacity of brown adipose tissue in the new-born animals is due to partial uncoupling of oxidative phosphorylation.

In the present work this "uncoupling" hypothesis has been extended in a study of cold adaptation in older animals.

Guinea pigs, at least 3 weeks old, were exposed to an ambient temperature of 6° C for periods ranging from 12 hrs to 2 months. In response to this thermal stress uncoupling of respiration linked phosphorylation in brown fat mitochondria took place after approximately 36 hrs of exposure. No "uncoupling" effect was detected in mitochondria from brain and liver tissue from the same animals.

We therefore propose an extension of the uncoupling hypothesis to apply to non-shivering thermogenesis of brown adipose tissue during cold adaptation in adult animals as well as in the neo-natal state previously investigated.

References

PEDERSEN, J. I., E. N. CHRISTIANSEN and H. J. GRAV, *Biochem. biophys. Res. Commun.* 1968. 32. 492—500.

C 10

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Arteriovenous Anastomoses in Temperature Regulation

By

L. Vanggaard

In mammals maintenance of a constant body temperature is ultimately dependent upon cooling of the blood in the body surface and subsequent transport of the cooled blood to the body core In the clothed human being the effective cooling surface is small, confined to the acral parts, hands, forearms and head In these areas the arteriovenous anastomoses play an important role in regulating local blood flow In this respect the clothed man resembles the furred animals

By means of thermocouple measurements under neutral thermal conditions it was observed that the skin temperatures of fingers and toes fluctuate synchronously while the skin temperatures over the rest of the body remain nearly constant. The synchronized changes in the skin temperatures of fingers and toes indicate a common regulatorly mechanism.

During spontaneous fluctuations the maximal steepness of the downward slopes in skin temperature is equal to that observed after stop of the blood flow in the area by an inflatable cuff applied to the forearm This shows that these fluctuations reflect a drastic decrease in blood flow and harmonizes with the idea of arteriovenous anastomoses as on—off shunts Plethysmographic measurements showed flows changing by a factor of 7—10 in the fingers in accordance to the results of others

Under neutral thermal conditions a longitudinal temperature gradient along the hands and forearms was found the fingertips invariably being warmest Furthermore the temperature of skin over the superficial veins of the hand and forearm was higher than that of the adjacent skin (as recorded by Vanggaard 1942) and followed the temperature fluctuations in the fingers with an amplitude decreasing proximally This temperature pattern was further visualized by an infrared scanner (AGA)

In resting persons under neutral thermal conditions the rate of heat loss from the superficial veins was estimated—from plethysmographic flow determinations and skin temperature differences along the veins—to 15—20 % of the basal heat production Fluctuations of considerably smaller magnitude are considered sufficient to maintain the regulation of heat control of the body under such conditions

References

Burton A. C *Amer J Physiol* 1939 *127* 437—453

Vanggaard Th Thesis Munksgaard Kbh 1941

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Short-Term Habituation and Dishabituation of the Sensory Evoked Response in Man

By

H. Fruhstorfer, T. Järvilehto and P. Soveri

Quantitative studies in six subjects have shown that the amplitude of the human vertex potential elicited by a group of eight identical clicks (1 group/100 sec, 1 click/1 sec) decreases rapidly and reaches a stable level at the third stimulus. For the prominent negative response component N_{1b} (mean peak latency 114 msec) this level amounts to 5.4% (s = 3.6%) of the initial value (30.5 µV, s = 10.6 µV). At a slower stimulation rate (1 click/3 sec) the amplitude decrease is less rapid and less pronounced, at the fifth stimulus N_{1b} reaches a stable level at 12.5% (s = 5.7%) of the initial value. *With both stimulation rates the amplitude decrease follows closely a* negative exponential course. In order to find out whether this response decrease which is characteristic for short-term habituation, meets also other criteria of habituation (Thompson and Spencer 1966), the interaction between a repeatedly presented stimulus of one modality and a single stimulus of another modality was examined.

In 20 sessions two subjects received groups of seven stimuli (40 groups/session, 1 group/60 sec, 1 stim/sec). The stimuli were clicks (C) of 60 dB above subjective threshold and shocks (S) to the median nerve just above motor threshold. In 10 sessions the order of the stimuli in the groups was CCCCSCC, in the other 10 SSSSCSS. *The vertex responses were recorded from Cz-Fpz and selectively sum*mated in a LINC computer according to their order in the groups.

With the presentation of the first 4 stimuli of either modality the amplitude of the vertex response decreased rapidly, time course and extent of the decrement depending on the subject and on the modality. Simultaneously with the amplitude change a slight latency reduction occurred. The fifth stimulus of different modality evoked a response which was always smaller than the non-habituated response to the same stimulus ($p < .01$, two-sided rank-sum test), but always bigger than the habituated response to this stimulus ($p < .01$). The amplitude decrease was more pronounced in the negative components N_{1b}, reaching on the average 37.8% (s = 14.4%) of its *non-habituated size*. Similarly the latency of N_{1b} was significantly reduced ($p < .01$). In the SSSSCSS condition the sixth stimulus response was slightly bigger than the fourth one due to an increase of N_{1b}.

These results show that there is strong cross-modal interaction between somatosensory and auditory stimuli when eliciting the unspecific vertex response in man.

This interaction results in partial stimulus generalization of habituation, which explains the finding that neither click nor shock produced any significant dishabituation.

References

THOMPSON, R F and W A SPENCER, *Psychol Rev* 1966 73 16—43

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The Effect of Noradrenaline on the Direct Cortical Response of Asphyxiated Newborn Rabbits

By

J BOETHIUS, T BRUNDIN and N A PERSSON

The central nervous system of newborn and fetal mammals can withstand the effects of asphyxia to a greater extent than that of the adult This ability to withstand asphyxia is mirrored by the resistance of the direct cortical response (DCR) as demonstrated in fetal lambs (Meyerson 1964) Since in fetal and newborn mammals asphyxia causes release of large amounts of noradrenaline from the preaortal para ganglia (Brundin 1966) and from the adrenal medulla (Comline and Silver 1958), the present study was undertaken to find out if noradrenaline affects the asphyxia resistance of the DCR of newborn rabbits

The rabbits were tracheotomized under ether anesthesia, immobilized with Flaxedil and ventilated artificially A unilateral craniotomy was made and the DCR was led off from the exposed cortical surface Immediately before asphyxiation each experi mental animal received a large dose of noradrenaline (80—200 ng/g b w s c) and the controls got corresponding volumes of saline

It was found that the resistance of the DCR of the untreated animals was similar to that which has been previously found for the adult, *i e* the DCR being blocked within 3 1/2 min After administration of noradrenaline the DCR became more resistant and could be evoked for 8—17 min after the onset of asphyxia

In view of the large doses of noradrenaline used in the present experiment it seems reasonable to assume that noradrenaline had entered the brain tissue and that the observed effect of noradrenaline is due to some direct action on the cells of the central nervous system The effect of noradrenaline might however also be caused by general circulatory and metabolic actions of the hormone, and it is not possible at present to distinguish between the various mechanism by which noradrenaline could cause asphyxia resistance of the DCR

References

Brundin, T, *Acta physiol scand* 1966 *70* Suppl 290
Comline, R S and M Silver, *Nature* (Lond) 1958 *181* 283—284
Meyerson, B A, *Acta physiol scand* 1964 *62* 489—490

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Tension and Contraction Velocity of Single Motor Units of the Stapedius Muscle

By

E Teig

Since the stapedius and the tensor tympani muscles are capable of delicate and swift variations in tension (Carmel and Starr 1963), their motor units are probably of small size and capable of fast contraction and relaxation Counts of the nerve and muscle fibres have shown the motor units of these muscles to be smaller than in most other skeletal muscles (Malmfors and Wersall 1960, Blevins 1967) Wersall (1958) calculated the twitch tension of the average stapedius motor unit in the rabbit to be between 8 and 70 mg but no direct measurements exist

In the present study the tension and contraction properties of individual stapedius motor units have been measured in the rabbit in response to threshold stimulation of the facial nerve The muscle was attached to a transducer with an output of 0 2 mV/mg The distortion of the signal by background mechanical noise, mainly due to

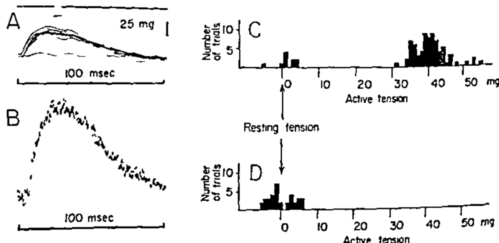


Fig 1 A All-or nothing contractions of a single motor unit B Average of 91 contractions of the same unit C Histogram of the tension developed by the same motor unit as in A and B to just threshold stimulation (91 successes and 9 failures) D Effect of subthreshold stimulation

the heart beat was counteracted by averaging 50—200 contractions (Fig 1B) The signal was digitalized by a voltage-to-frequency converter which fed an electronic counter, and the tension curve was integrated for 5 msec at the peak of the contraction (Fig 1A gating pulse upper line) Fig 1C shows the integrated values obtained on 100 consecutive trials The peak at resting tension (Fig 1C D) represents the baseline variation and the peak at 40 mg the force of a single motor unit

The twitch tension of different motor units ranged from 13 to 90 mg Nine different motor units showed contraction times between 19 and 31 msec, and half relaxation times from 22 to 64 msec Supramaximal stimulation gave twitch contraction times between 21 and 24 msec Hence the stapedius may be classified as a fast muscle The wide range in contraction times and twitch tension of the stapedius motor units is similar to that found in other fast muscles (Wuerker *et al* 1965, Burke 1967)

References

Blevins C E *Arch Otolaryng* 1967 *86* 136—142
Burke R. E. *J Physiol* (Lond) 1967 *193* 141—160
Carmel P W and A Starr *J Neurophysiol* 1963 *26* 598—616
Malmfors T and J Wersall *Acta morph neerl scand* 1960 *3* 157—169
Wersall R *Acta Otolaryng* 1958 Suppl 139
Wuerker R. B A M McPhedran and E Henneman *J Neurophysiol* 1965 *28* 89—99

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Renal Handling of Galactose in the Cat

By

S Nybo Rasmussen

Extrahepatic elimination of galactose occurs mainly in the kidney Urinary excretion as well as metabolic utilization may be of significance The mechanism of urinary excretion of galactose is not clearly understood (Tygstrup 1961 Tengstrom 1968) The total renal capacity for metabolic utilization has not been measured and its significance for the reabsorption has not been evaluated

Clearance measurements were done at practically constant plasma concentrations of galactose between 60 and 1900 mg/liter In the concentration range 60 to 400 mg/liter renal blood flow was simultaneously measured and the rate of utilization in the kidneys was calculated as the blood flow times the renal arteriovenous deficit of galactose minus the excretion rate The urine never was free of galactose and urine/plasma concentration ratios below one pointing to uphill transportation were never observed At low filtered loads the reabsorption fraction (reabsorption rate/filtra

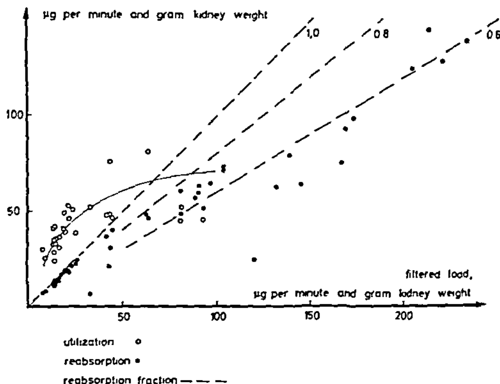


Fig 1 The relationship between filtered loads of galactose and the rates of galactose reabsorption and metabolic utilization

tion rate) was constant and close to one (0 9) At higher loads the reabsorption fraction decreased apparently converging towards 0 6

At low filtered loads the utilization rate was considerably higher than the reabsorption rate At increasing filtered loads the utilizations approached maximum values between 50 and 80 µg galactose/minute gram kidney weight.

Push-flow experiments (Aukland and Kjekshus 1966) indicated that reabsorption of galactose occurs exclusively in the proximal tubular system. When, at low plasma concentrations, the utilization rate exceeds the reabsorption rate the intracellular concentration of galactose may stay low and this may promote diffusion into the cells and account for the high reabsorption fractions found At higher concentrations, where the reabsorption rate exceeds the utilization rate reabsorption is probably increasingly due to transcellular back-diffusion.

References

Aukland K. and J Kjekshus *Amer J Physiol* 1966 *210* 971—979
Tengström, B., *Scand J clin Lab Invest* 1968 *4* 321—326
Tygstrup, N, *Acta physiol scand* 1961 *51* 263—274

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Mechanisms in the Maintenance of Glomerular Filtration Rate During High Urine Flow

By

J Kjekshus, E Loyning and F Kiil

During osmotic diuresis, glomerular filtration rate (GFR) remains constant, despite a large increase in proximal tubular pressure (Gottschalk 1964) Maintenance of GFR probably implies a reduction in preglomerular vascular resistance In experiments on anesthetized dogs, we have examined the possibility that the reduction in preglomerular vascular resistance is a consequence of renal autoregulation Our main findings are summarized in Fig 1

A nearly linear relationship between renal perfusion pressure and metered renal blood flow (RBF) was observed at 1) high pressure in the renal pelvis, or 2) at normal pelvic pressure, but high urine flow (>15 ml/min) induced by ethacrynic acid or furosemide (B), Fig 1 The upper curve (C) was obtained during expansion of extracellular volume with 1) isotonic or hypertonic mannitol, or 2) infusion of isotonic saline at 30—40 ml/min At all perfusion pressures, RBF was much higher than during control conditions (lower curve, A), but it still displayed autoregulation except at the highest rates of infusion when the flow/pressure relationship become linear Comparable dilution of blood by infusion of saline into the renal artery caused less increase in RBF, indicating that the increase in RBF during expansion of extracellular volume was not solely a consequence of reduced blood viscosity

As both high ureteral pressure and high urine flow dilate the autoregulating elements, the present studies support the hypothesis that transmural pressure of the afferent arterioles is the variable controlled by the autoregulating mechanism In con-

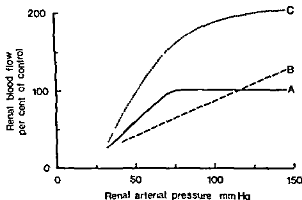


Fig 1 Flow/pressure relationship during (A) control conditions (B) high diuresis (ethacrynic acid) or during ureteral occlusion (renal pelvic pressure 45 mm Hg), and (C) expansion of extracellular volume with saline (40 ml/min)

trast intravenous infusions of mannitol or saline increased RBF without influencing autoregulation untill intrarenal pressure rose as a consequence of high urine flow

References

GOTTSCHALK, C W, *Circulat Res* 1964 *15* (Suppl I) 110—114

C 16

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Water Balance in the Pupa of the Silk Moth, Antheraea Pernyi

By

H J FYHN and T SÆTHER

Larvae and adult insects can regulate the osmolality of their haemolymph (NEMENZ 1960, SCHOFFENIELS 1960) but osmotic regulation during metamorphosis has not been investigated As the pupa is a closed system for non volatile substances, metabolites and excretory products have to remain inside the pupa This accumulation may lead to changes in haemolymph osmolality

During the transformation from larva to imago, we found a weight decrease of 85 %, most of it due to loss of water The weight decrease during pupal life proper is only 10 %, showing that loss of water was kept at a minimum during this period The simultaneous loss of dry weight was greater than the loss of water during

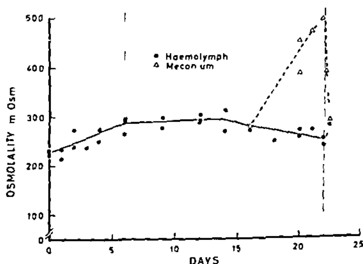


Fig 1 Changes in osmolality of haemolymph and meconium during metamorphosis Pupation and emergence are indicated by broken lines at day 6 and day 22, respectively Each point represents measurements on one animal

the pupal stage, and consequently, the relative water content increased from 77 % to 81 %

By calculating the change in absolute water content during the pupal stage, a decrease of 6 % was found In the pupa as a whole, one should therefore expect a small concentration of the solutes No such concentration was observed in the haemolymph, as shown in the figure

However, the pupal hindgut developed and functioned during the last part of the pupal stage, and its contents, meconium, became highly concentrated At emergence, its osmolality was twice that of haemolymph, as shown in the figure

We conclude that the pupa has the ability to withdraw osmotically active particles from the circulating haemolymph and concentrate them in the hindgut This, in addition to the water conservation, is interpreted as a regulation of the haemolymph osmolality

References

NEMENZ, H, *J Insect Physiol* 1960 4 38—44
SCHOFFENIELS, E, *Arch int Physiol Biochim* 1960 *68* 507—508

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The R/V Alpha Helix Bering Sea Expedition, The Scripps Institution of Oceaneography, La Hoya, U S A

Blood Flow in the Interdigital Web of Sea Gull at Low Temperatures

By

K JOHANSEN and K H TONNESEN

The sea gull is able to rest on ice at temperatures below the freezing point The purpose of the present study was to investigate how the skin avoids freezing without intolerable heat loss from the bird This mechanism must partly be connected with the blood flow of the foot

Sejrsen (1968) demonstrated the existence of a gas tight membrane in the skin of man This membrane allows only a fraction of a gas of any partial tension to enter by diffusion When a chamber with a high gas tension is placed on the skin for 6 minutes a fraction of the gas will diffuse into the skin The gas will be cleared predominantly by the blood flow as this process is much faster than the diffusion through the skin By using a radioactive gas the skin blood flow can be measured

In the present study the existence of a gas tight membrane in the web of the sea gull was substantiated by demonstrating negligible clearance of 85 Kr from the interdigital web during arterial occlusion

The blood flow was calculated from the externally detected clearance curve of 85 Kr at external temperatures between —1 5° C and 27° C The temperature of the web varied between 4° C and 27° C, the blood flow varied between zero and 18 ml/100 g min A positive correlation was found ($r=0\ 98$, $p<0\ 001$, $N=8$)

In an experiment at a low relatively constant temperature of the foot (4° C) a fluctuation in the blood flow was observed In successive periods of 10—30 minutes the blood flow level changed from 1 8 ml/100 g min to zero and then increased to 3 ml/100 g min This observation suggests a mechanism by which the bird avoids excessive heat loss from the body and frostbites of the feet

References

SEJRSEN P *J appl Physiol* 1968 24 211

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Blood Flow Through Muscles during Heavy Rhythmic Exercise

By

B FOLKOW P GASKELL and B A WAALER

rhythmically working muscles blood vessels will be compressed during each con phase This will cause temporary reduction in venous blood content and phasic lowering of local venous pressure The reduction in venous pressure can apparently be very marked when transmural pressure is high such as in a dependent limb where gentle movements are known to reduce local venous pressure considerably A reduction in local venous pressure must again imply an increase in regional perfusion pressure During heavy rhythmic exercise where vessels are already maximally dilated a maintained reduction in mean muscular venous pressure would represent an important gain in perfusion pressure and consequently in flow However it is not known if the huge inflow in such a situation may refill the rhythmically emptied veins so rapidly that no significant gain in perfusion pressure is obtained

Introductory experiments in anesthetized cats showed that mean pressure in a small calf muscle vein could be maintained at a lowered level throughout a period of heavy rhythmic exercise The degree of pressure reduction depended on the frequency and relative duration of the single muscle contractions

A model study of the flow situation in exercising muscles was then performed on the isolated calf muscles of the cat prepared according to Kjellmer (1964) Phasic flow was recorded with an electromagnetic flowmeter on the arterial or on the venous side of the preparation Arterial inflow and venous outflow pressure were recorded Transmural pressure could be increased by at least 25 mm Hg by lowering the isolated limb in relation to the animal Rhythmical contractions were achieved by

supramaximal sciatic nerve stimulations once per sec for 250 msec at 40—60 imp/sec

Each contraction phase caused a virtual cessation of blood inflow into the calf muscles whilst at the same time blood was ejected at the venous side During the relaxation phase inflow became maximal whereas there was then only a very small outflow from the preparation —The net blood flow through muscles performing heavy rhythmic exercise could be considerably increased by lowering the exercising limb and thus elevating transmural pressures In such a dependent limb with maximal vasodilatation average flow through the muscles was about the same *during* a period of rhythmic contractions as in the post exercise situation with free flow A substantial gain in effective perfusion pressure and in flow can thus apparently be achieved in a dependent limb performing heavy rhythmic exercise

References

KJELLMER I *Acta physiol scand* 1964 62 18—30

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Effects of Growth Hormone on Amino Acid Transport and Protein Synthesis in the Isolated Working Rat Heart

By

K. AHREN A HJALMARSON and O ISAKSSON

Both size and mechanical performance of the rat heart are reduced after hypophysectomy (Beznak 1954) It has also been shown (Beznak 1964) that treatment of hypophysectomized rats with a combination of growth hormone (GH) and thyroxine restore all basal cardiovascular parameters to normal

In the present study rat hearts were perfused by a new technique developed by Morgan *et al* (1965) where in contrast to the classical Langendorff preparation the perfusate is introduced into the left atrium and pumped by the left ventricle against a hydrostatic pressure head Hearts from hypophysectomized rats perfused for various periods with Krebs bicarbonate buffer containing glucose (1 mg/ml) were found to have significant lower weight and showed lower systolic pressure cardiac rate, cardiac output and coronary flow than hearts from normal rats Studies have been started in order to analyse the mechanism(s) for these differences between hearts from normal and hypophysectomized rats As a first part of this analysis the acute *in vitro* effect of GH on amino acid uptake studied with the non utilizable amino acid *a* aminoisobutyric acid (AIB) and on the incorporation of phenylalanine ^{3}H of the perfused working rat heart was studied A GH concentration of 25 μg/ml in the perfusate significantly increased the rate of accumulation of AIB-^{14}C of the heart muscle

TABLE I Effects of GH on the accumulation of AIB-^{14}C and on the incorporation of phenylalanine-^{3}H into the protein of the left ventricle of the isolated working heart from hypophysectomized rats

Labelled substance	Perfusion time (min)	GH 25 μg/ml	Distribution ratio	Incorporation
AIB	30	−	1 97±0 20 (8)	
			$p<0\ 001$	
	30	+	3 37±0 25 (9)	
	60	−	4 77±0 25 (4)	
			$p<0\ 01$	
	60	+	6 94±0 50 (4)	
Phenylalanine	120	−	5 74±0 28 (4)	10624±312 (7)
			$p<0\ 01$	$p<0\ 001$
	120	+	4.24±0 18 (4)	13139±597 (7)

given

(Table I) The same GH concentration also increased the rate of incorporation of elled phenylalanine into the protein of the perfused heart from hypophysectomized al

This stimulatory effect of GH on the uptake and incorporation of amino acids may be of physiological significance for maintenance of normal cardiac protein synthesis

References

BEZNAK M *J Physiol* (Lond) 1954 *124* 44—63

BEZNAK M *Circulat Res* 1964 *15* Suppl 2 141—150

MORGAN H E J R NEELY R E WOOD C LIEBECQ H LIEBERMEISTER and C R PARK *Fed Proc* 1965 *24* 1040—1045

C 20

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The Effect of Cooling on Liver Metabolism

By

J A LARSEN

In experiments on cats anesthetized with chloralose (40 mg/kg) it was observed that cooling of the animal either by means of an air stream or by intra abdominal cooling decreased the apparent elimination rate of glycerol

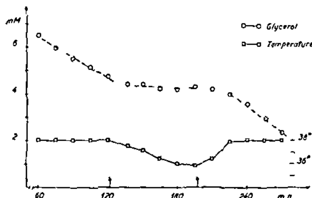


Fig 1 The elimination curve of glycerol during continuous infusion of glycerol (99 μmoles/kg/min) The *arrows indicate time* of cooling and heating Ordinate glycerol concentration in plasma (mM/l) Abscissa time (minutes)

The elimination rate was decreased by 30—50 per cent within a few minutes and remained constant during the rest of the cooling period Upon heating the depressive effect appeared to be reversible (see Fig 1) The elimination of ethanol and galactose was likewise decreased by cooling but not to the same extent as with glycerol

The cooling was not accompanied by reactions normally seen in unanesthetized animals (Masoro 1966) Thus there was no observable muscle shivering and curare did not block the response to cooling

The oxygen uptake remained constant and there was no change in the concentration of plasma FFA There was no sign of increased production of adrenaline or noradrenaline and α and β blockers did not change the response to cooling After dissection of the nerve plexus in the liver hilus followed by applicance of local anesthesia, the depressive effect was still observed

The *metabolism of glycerol, ethanol and galactose is dependent on the function of* the liver and the observed effect of cooling therefore seems to be located in this organ The way in which cooling exerts this effect is, however still obscure

References

Masoro E. J *Physiol Rev* 1966 *46* 67—101

C 21

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Microspectrophotometric Study of the Compound Rhabdom in the Moth (Manduca sexta) Eye

By

S D Carlson and B Philipson

In the superposition eye of this nocturnal moth, the photoreceptor cells cluster to make up thousands of 8 celled units (retinulae) Each cell contains a rhabdomere which is a rank of microvilli containing visual pigment These microvilli oppose those of the adjacent cell so there is no spatial separation between neighboring rhabdomeres as in the apposition eye of the fly

This study s objective was to determine if absorption spectra of visual pigments could be resolved when the pigment containing sites of adjacent cells are continuous These data also serve as a comparison to the electrophysiological recordings obtained from single cells in the moth (Boethius *et al* 1968)

Cryostat sectioned eye tissue was mounted in glycerin between quartz glasses and the absorption of the visual pigments was measured with a dual beam recording microspectrophotometer (Zeiss UMSPI)

Absorption maxima were found principally in the following regions 510—520 nm 480—490 nm and 450—460 nm Few records showed a maximum in the near UV at 340—350 nm There was also some indication of a maximum in the 540—550 nm area These maxima are in good agreement with those derived from spectral efficiency curves of individual photoreceptor cells determined by microelectrode technique for *Manduca sexta* (Boethius *et al* 1968) Bleaching experiments revealed two photolabile pigments with maxima at about 445 and 485 nm During these bleaching losses an absorption build up occurred in the near UV

Measurements in various quadrants of the retinula demonstrated a variation in maxima which makes it likely that adjacent cells may have different colour receptor properties Thus it is possible that all visual pigments are present in an 8 celled cluster of photoreceptor cells It is interesting that the retina of a night flying moth contains several photopigments with differing color sensitivity
cluster of photoreceptor cells Because of the small size of the measuring beam (1 μ in diameter) and only one maximum per scan there is apparently one visual pigment per photoreceptor cell

References

Boethius J S D Carlson G Hoglund and G Struwe *Acta physiol scand* 1968 In press

C 22

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Effect of Smooth Muscle Contraction on Aortic Diameter and Aortic Baroreceptor Activity

By

HARALD AARS

The present study was undertaken to examine whether aortic baroreceptor activity is modified by the state of contraction of aortic smooth muscle

The investigations were carried out in rabbits anesthetized with chloralose and urethane Baroreceptor activity was recorded in the whole left aortic nerve, and quantitated by rectification and integration (Aars and Leraand 1968a) Blood pressure was measured in the right common carotid artery For recording of diameter in the ascending aorta, piezoelectric crystals were glued to aorta one week prior to the experiments (Aars and Leraand 1968b) Nerve activity and aortic diameter were recorded at various blood pressure levels produced by stepwise withdrawal and reinfusion of blood through the right jugular vein before and during infusion of noradrenaline The doses of noradrenaline (1—2 μg/kg min) were selected so as to give only 20—40 mm Hg rise in control diastolic blood pressure

In most cases the relationship between arterial blood pressure and activity in the aortic nerve was nearly the same before and during infusion of noradrenaline Noradrenaline produced about 5 % reduction in aortic diameter, but pulse pressure and rate of change of pulsatile diameter variations increased When both diastolic and pulsatile diameter variations were taken into account, the relationship between aortic diameter in the receptor area and aortic nerve activity was found to be largely independent of the degree of smooth muscle contraction Aortic nerve activity increased considerably after injection of phenoxybenzamine (1 mg/kg), due to a 10—15 % increase in diameter but the relationship between diameter and receptor activity changed very little

It is concluded that the state of contraction of the aortic smooth muscle whether increased by moderate doses of noradrenaline or reduced by phenoxybenzamine, did not significantly influence the relationship between aortic diameter and activity in the aortic baroreceptor nerve

References

AARS H and S LERAAND *J appl Physiol* 1968a 24 416—418
AARS H and S LERAAND *Acta physiol scand* 1968b 73 22—23A

C 23

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Descending Monosynaptic Connexions to Spinal Border Cells

By

F Baldissera and F Weight

The spinal border cells (SBC) have recently been identified as cells of origin of ventral spinocerebellar axons (Burke *et al* 1968) Monosynaptic EPSPs and/or IPSPs may be evoked in the SBC by stimulation, at low thoracic level, of ipsilateral descending fibres The purpose of the present investigation was to determine the origin of ventral descending fibres monosynaptically connected to SBC

In chloralose anesthetized cats we have recorded intracellularly from 48 SBC, identified by their antidromic activation from the contralateral thoracic cord and by their location in the lateral part of the ventral horn Tungsten electrodes were inserted ipsilaterally in Deiters' nucleus and in the medial longitudinal fascicle (MLF), both known to project directly on motoneurones and ascending tract cells in the lumbar cord (Grillner *et al* 1968)

In 16 cells stimuli in Deiters' nucleus (max intensity 100 μA) evoked EPSPs with a segmental latency ranging from 0 4 to 1 1 msec, thus compatible with a monosynaptic linkage Stimulation of the MLF gave EPSPs with segmental latencies in this range in 13 cells Convergence from these two sources was observed in 6 cells Short latency IPSPs were also evoked in the SBC from the brain stem In four cells stimulation of the MLF produced IPSPs with a segmental latency of less than 1 1 msec In two of them the segmental latency was 0 63 and 0 75 msec respectively thus strongly suggesting a monosynaptic linkage from fast conducting fibres

It is concluded that three ventral pathways of supraspinal origin have monosynaptic connexion with SBC two of them are excitatory and one inhibitory Recently Wilson (1968) reported monosynaptic inhibitory connexions from the brain stem to cervical motoneurones Our results give the first evidence of long inhibitory axons descending from the brain stem to the lumbar enlargement

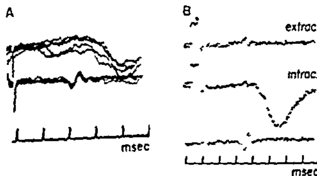


Fig 1 IPSP evoked in a spinal border cell by MLF stimulation A intracellular potential (upper trace) and the descending volley (lower trace) recorded from the lateral surface of the same spinal segment in which the cell was located B 40 responses averaged on a CAT 1000 Upper trace gives the extracellular field potential Calibration in averaged records 0 5 mV

References

BURKE, R, A LUNDBERG and F WEIGHT, *Acta physiol scand* 1968 *74* 16A
GRILLNER, S, T HONGO and S LUND, *Brain Res* 1968 *10* 259—262
WILSON, V J and M YOSHIDA, *Brain Res* 1968 *11* 691—693

C 24

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The Noradrenergic Control of the Resting Activity in Static γ-Motoneurones and Its Relation to the Tonic Stretch Reflex

By

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C 25

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Two Inhibitory Reflexes to the Abdominal Stretch Receptors of the Crayfish

By

J K S JANSEN, A NJÅ and L WALLOE

Two efferent nerve fibres the thick and the thin 'accessory' nerves, establish synaptic contacts with the soma-dendritic regions of the abdominal stretch receptors of the crayfish (Alexandrowicz 1967) The thick accessory nerve is inhibitory (Kuffler and Eyzaguirre 1955) and can be reflexly activated from the stretch receptors of the same and neighbouring segments (Eckert 1961, Fields Evoy and Kennedy 1967)

By stretch activation of the slowly adapting receptor or by comparable electrical stimulation of the dorsal nerve we have regularly found reflex activation of two efferent spikes of different amplitudes in the dorsal nerves of the same and the neighbouring segments Both reflexes can have latencies as short as 30 to 40 msec and both give a maintained response with only little adaptation to a steady state input

There is a certain difference in the distribution of the activation of the large and the small efferent spikes The large spike is most effectively activated ipsilaterally while the small spike is more prominent in crossed reflexes The nature of the reflexly activated spikes can be determined by their effect on the discharge of the receptor The large as well as the small reflex spike consistently prolonged the interval between receptor impulses in which the

reflexes allowed independent confirmation of the inhibitory effect of both the large and the small reflex spikes

In conclusion, the present experiments confirm Eckerts (1961) description of a reflex activation of the thick accessory nerve In addition a stretch receptor input also activates the thin accessory nerve Its effect is inhibitory and the distribution of the two reflexes is partly overlapping The functional significance of this inhibitory feedback to the stretch receptors is at present unknown

References

ALEXANDROWICZ J S *Biol Revs* 1967 *42* 288—326
ECKERT, R. O, *J Cell comp Physiol* 1961 *57* 149—162
FIELDS H L. W H EVOY and D KENNEDY, *J Neurophysiol* 1967 *30* 859—874
KUFFLER S W and C. EYZAGUIRRE, *J gen Physiol* 1955 *39* 155—184

C 26

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Influence of Prolonged Overhydration and Dehydration on Vasopressin Content and Neurosecretory Material in the Hypothalamo-Neurohypophysial System of Rats

By

H VILHARDT

Previous studies have shown that dehydration reduces the amount of stainable neurosecretory material (NSM) in the neurohypophysis of rats (Ortmann 1951) There is some disagreement concerning changes in hormone contents as measured by bioassay under these circumstances

In a few earlier studies the contents of NSM have been studied in animals which have been overhydrated The results vary from no changes at all to a considerable augmentation There are no reports of the contents of hormone in overhydrated animals In the present study rats were subjected to different degrees of hydration varying from 10 days of dehydration to 5 days of overhydration The total contents of vasopressin in the neurohypophysis and in the hypothalamus was determined by the rat blood pressure method and in parallel with this sections of neurohypophyseal tissue were stained with paraldehyde fuchsin to attempt demonstration of quantitative changes in the NSM

It was found that with progressive dehydration there was a decrease of vasopressin content in the neurohypophysis whereas the amount of hormone in the hypothalamus was unchanged in the beginning of the dehydration period but showed a considerable fall at the end of 10 days dehydration In parallel with this there was observed

a decrease of the neurohypophyseal NSM, the gland being totally depleted after 10 days

During overhydration there was a considerable accumulation of vasopressin in the neurohypophysis Microscopical examination of the neurohypophysis of these overhydrated animals showed a considerably increased storage of NSM after 5 days

References

ORTMANN R, Z *Zellforsch* 1951 *36* 92—140

C 27

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Effect of Metabolic Inhibitors on the Release of Vasopressin in vitro

By

N A THORN and J WARBERG

According to Douglas and Poisner (1964), the most likely process involved in the release mechanism for vasopressin is reverse pinocytosis of neurosecretory granules A different hypothesis put forth by Thorn (1965) assumes a complex dissociation between vasopressin and carrier protein to be the essential feature

One of the experimental facts forming the basis for Douglas and Poisner's concept was the finding that metabolic inhibitors cause an inhibition of the release of vasopressin from neural hemilobes in vitro caused by excess calcium (Douglas *et al* 1965) Since rather few experiments were made and since these results are important for the concept of the mechanism of release the release of vasopressin from isolated rat neurohypophyses was studied using a Ca free K_2SO_4 Locke solution to which Ca was added, or a modified Locke solution to which excess K was added When the hemilobes had been incubated in the Ca free Locke medium or the modified Locke medium for 40 min. in the presence of inhibitor (2 4 dinitrophenol (0 5 mM) or iodoacetate (3 mM)) the release caused by addition of Ca (respectively K), was inhibited considerably However during the 40 min baseline period with incubation in DNP-containing medium, the baseline rate of release was considerably higher than in control experiments containing no DNP No certain conclusion concerning this latter phenomenon could be drawn in the experiments with iodoacetate

The small release of hormone after stimulation following a 40 min incubation with metabolic inhibitor may be due to a depletion of 'easily releasable' hormone Alternative explanations are interference with a hypothetical active transport of calcium into the cells or inhibition of an energy-dependent step involving reverse pinocytosis

References

DOUGLAS W. W. and A. M. POISNER, *J. Physiol.* (Lond.) 1964 *172* 1—18
DOUGLAS W. W., A. ISHIDA and A. M. POISNER, *J. Physiol.* (Lond.) 1965 *181* 753—759
THORN, N. A., *Acta endocr.* (Kbh.) 1965 *50* 357—364

C 28

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Ouabain-Sensitive Carrier-Mediated Transport of Glucose in the Cerebral Ventricles of Cats

By

H. E. BRØNDSTED

The transport of glucose from blood into ventricular cerebrospinal fluid (CSF) in dogs is carrier mediated (Fishman 1964).

In order to study the transport in the opposite direction, artificial CSF containing C-14 glucose and glucose in different concentrations was perfused through the lateral and III^rd cerebral ventricles of cats. The outflow was collected from a catheter inserted into the cerebral aqueduct. By determination during steady state of the specific activity of glucose in the perfusate and rates of flow through the ventricles it was possible to determine the unidirectional flux of glucose from the ventricles to blood and brain ($J_{v \to b}$) and the unidirectional flux from blood and brain to the ventricles ($J_{b \to v}$).

The extraction of C-14 glucose from the perfusate decreased from 18.6 per cent to 13.9 per cent of the inflow activity when perfusate glucose concentration was increased from 1 mM to 34 mM. Addition of ouabain (5×10[illegible] M) to the perfusate reduced both of the unidirectional fluxes. The difference between $J_{v \to b}$ before and after addition of ouabain was found to be a saturable transport process. All effects of ouabain, i.e. reduced glucose transport and CSF formation, reduced outflow of sodium and increased outflow of potassium, could be imitated by complete replacement of potassium in the perfusate with equivalent amounts of sodium. Azetazolamide in the perfusate (10[illegible] M) reduced CSF formation without reducing the fluxes of glucose. This finding suggests that carrier mediated transport of glucose from blood to cerebral ventricles may be independent of CSF formation. Counter transport was demonstrated by addition of mannose or xylose to the perfusate whereby $J_{v \to b}$ was increased. This finding and the saturation phenomenon give strong evidence for carrier mediated transport of glucose in the cerebral ventricles.

References

FISHMAN R. A., *Amer. J. Physiol.* 1964 [illegible] 835—844

C 29

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Influence of Colchicine on Axoplasmic Transport of Amine Storage Granules in Rat Sympathetic Adrenergic Nerves

By

A Dahlstrom

Strong evidence for the existence of a proximo distal transport of amine storage granules in sympathetic adrenergic nerves has been presented (*e g* Dahlstrom 1965, Kapeller and Mayor 1969) The granules are probably manufactured in the perikaryon, transported distally at a rate of about 5—10 mm/h and have a life span in the nerve terminals of several weeks (Dahlstrom and Haggendal 1966)

So far, the mechanism of this fast proximo-distal transport of granules is unknown However it has recently been proposed that the neurotubules may take part in fast axonal transport (*cf* Schmitt 1968) Colchicine has been found to destroy neurotubules by combining with the subunit proteins (Schmitt 1968) Experiments by Kreutzberg (pers comm) indicate that colchicine may inhibit the fast transport of acetylcholinesterase in myelinated axons in rat sciatic nerve It thus was thought of interest to study the effect of colchicine also on granular transport in unmyelinated nerves The histochemical method for localization of catecholamines and serotonin was used in a preliminary study (Dahlstrom 1968)

A) Colchicine dissolved in saline was injected under the epineurium of the sciatic nerve unilaterally An equal volume of saline (5 μl) was injected in the contralateral nerve as a control Injection of 100 or 1000 μg of colchicine resulted in histochemically pronounced accumulations of green fluorescent material (noradrenaline NA) in bulgy axons above and partly within the area of injection Saline only or saline containing 10 μg of colchicine had no observable effect

B) Soaking the lumbar sympathetic ganglia (giving rise to the adrenergic axons in the sciatic nerve) with 100 or 1000 μg of colchicine in saline strongly increased the specific green NA fluorescence in both cell bodies and intraganglionic axons The axons became slightly enlarged filled with NA in all probability stored within storage granules If the sciatic nerves were ligated 24 hrs after this treatment no or very few accumulations were observed in contrast to saline treated rats where large accumulations of NA above the ligation were always seen

The results indicate that colchicine may cause interruption of the fast transport of storage granules Since colchicine has been demonstrated to destroy neurotubules, the present results suggest that the mechanism of transport of amine storage granules may be connected to the neurotubules

References

DAHLSTROM A., J Anat (Lond.) 1965 99 677—682
DAHLSTROM A. Europ J Pharmac 1968 5 111—113
DAHLSTROM A. and J HAGGENDAL, Acta physiol scand 1966 67 278—288
KAPELLER, K. and D MAYOR, Proc roy Soc B 1967 167 282—292
SCHMITT F O., Proc nat Akad Sci (Wash.) 1968 60 1092—1101

C 30

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The Release Reaction of Secretion

By

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C 31

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Is Noradrenaline an Inhibitory Transmitter to Motoneurones?

By

G TEN BRUGGENCATE and I ENGBERG

The question was raised when noradrenaline (NA) containing terminals of descending tracts were found in motor nuclei of the spinal cord by fluorescence microscopy (Dahlstrom and Fuxe 1965) The findings indicated that these terminals were in intimate contact with motoneurones Microelectrophoretic application of NA in the vicinity of single motoneurones has been reported to depress the extracellularly recorded firing in some of these neurones (Engberg and Ryall 1966 Weight and Salmoiraghi 1967 Facilitatory effects of NA on motoneurones have not been found. We have now investigated the action of NA on a small number of motoneurones using a technique which permits intracellular recording with simultaneous extracellular electrophoretic drug application with parallel micropipettes) The efficiency of the electrophoretic drug administration was judged by alternate ejections of NA and glycine from the multibarrelled extracellular micropipette Glycine proved as reported earlier Curtis et al 1968 to have a potent inhibitory action on motoneurones mostly hyperpolarizing their membranes and strongly increasing their membrane conductances Similar actions were never found with NA (Fig 1 whether the neurones responded to glycine ejections or not Of 30 cells where glycine had strong actions only 2 showed any reaction to NA a small reduction of the discharge evoked by skin nerve stimulation However in comparing the microelectrophoretic effects of NA and glycine one might consider the possibility that the potency of NA as a transmitter could be lower than that of glycine and thus the amounts of NA

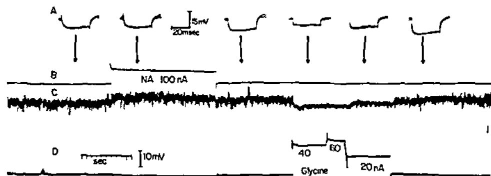


Fig 1 Effects on membrane conductance (records at A) and membrane potential (record C) of a motoneurone by microelectrophoretic application of noradrenaline (signal B) and glycine (signal D) Membrane conductance was measured by current pulses of 10 nA passed through the recording electrode

ejected could have been too small to act on a sufficient number of receptors even when comparatively strong electrophoretic currents were used The proximity of the drug pipettes to the cell somata appeared to be very important for evoking the glycine response since the distance between the intracellular and extracellular pipette tips had to be kept below some 30 μ It is possible therefore that the sensitivity of only a small part of the soma membrane was tested by the drug applications and that the absence of NA effects was due to a lack of NA receptors at the soma (into which the intracellular electrode is most likely to penetrate) Thus the present findings are compatible with the hypothesis of NA as an inhibitory transmitter to motoneurones only if its synapses are assumed to be located further out on the dendrites

References

CURTIS D R L HOSLI, G A R JOHNSTON and I H JOHNSTON *Exp Brain Res* 1968 *5* 235—258

DAHLSTROM A and K FUXE *Acta physiol scand* 1965 *64* Suppl 247 7—85

ENGBERG I and R W RYALL *J Physiol* (Lond) 1966 *185* 298—322

WEIGHT F and G C SALMOIRAGHI *Nature* (Lond) 1967 *213* 1229—1230

C 32

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GABA Synthesis in Rat Hippocampus Correlated to the Distribution of Inhibitory Neurones

By

F FONNUM and J STORM MATHISEN

When GABA is applied to cortical neurones by microiontophoresis its effect closely mimics the IPSP (Krnjević and Schwartz 1967) In brain GABA is synthesized from

L-glutamate by L-glutamate 1-carboxy lyase (GAD), which is predominantly localized in the nerve ending particles (Fonnum 1968) If GABA acts as an inhibitory *neurotransmittor its synthesis should take place within the inhibitory neurones*

The hippocampus regio superior (CA I) is characterized by a simple and regular structure were the inhibitory and excitatory synapses are spatially separated so that they may be isolated by microdissection The pyramids are concentrated in a single layer with the dendrites extending through the rest of the gray matter They are excited by axo-dendritic synapses, but receive recurrent inhibition through synapses located on their somata (Andersen *et al* 1963)

Samples (0 3—2 μg) were dissected from freeze dried sections (Lowry 1953) GAD was determined largely according to Albers and Brady (1959) In Fig 1 the results are presented relative to the mean activity of strips cut through all layers The s e m as well as the number of determinations and animals are indicated A drawing of a horisontal section stained with the Koelle method for AChE shows the area from which samples were obtained

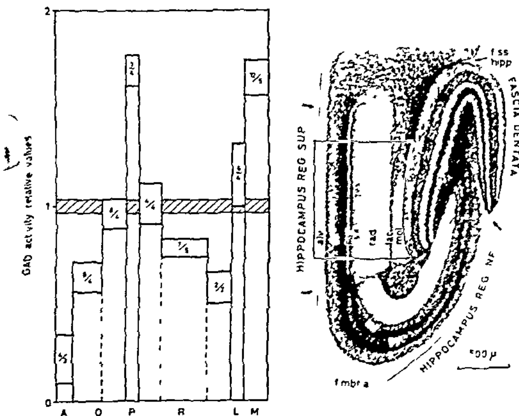


Fig 1 Histogram showing GAD activity in hippocampus reg sup Height of block on top of each column equals 2 x s e m Figures inside blocks indicate number of determinations and animals Hatched block represents strips running perpendicularly through all layers Samples were obtained from the area circumscribed in the drawing which is based on a horizontal section stained with the Koelle method for AChE

The concentration of GAD around the pyramids with gradual decline towards both sides closely parallels the distribution of the basket cell system GAD also shows a high activity throughout str moleculare and in contrast to AChE is not restricted to its caudal parts In this layer GAD and AChE are thus hardly concentrated in the same nervous elements Str moleculare contains neurones with short axons not unlike the basket cells, but no inhibitory system has hitherto been demonstrated in the layer

References

ALBERS R. W and R O BRADY, *J biol Chem* 1959 *234* 926—928
ANDERSEN P J C ECCLES and Y LØYNING *J Neurophysiol* 1964 *27* 592—607
FONNUM F, *Biochem J* 1968 *106* 401—412
KRNJEVIĆ K and S SCHWARTZ, *Exp Brain Res* 1967 *3* 320—336
LOWRY, O H, *J Histochem Cytochem* 1953 *1* 420—428

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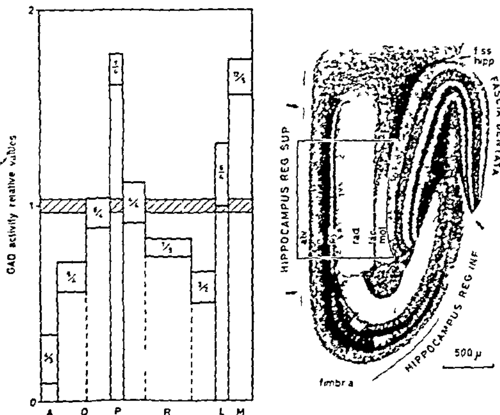


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Acta physiol. scand. 1969. 76. 257—269

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Innervation by Nerve Implants of "Fast" and "Slow" Skeletal Muscles of the Rat

By

S Fex and Isa Jirmanová

Received 30 August 1968

Abstract

Fex S and I Jirmanová *Innervation by nerve implants of "fast" and "slow" skeletal muscles of the rat* Acta physiol scand 1969 76 257—269

The time course of innervation by nerve implants of the fast contracting flexor digitorum longus and the slow contracting soleus muscles were studied by the recording of isometric

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days were required for the implanted nerve to innervate the muscle Implantation of the nerve which normally innervates the flexor digitorum longus muscle into the soleus altered the contraction and relaxation speeds of the soleus muscle towards those of a fast contracting muscle the change being apparent as soon as 4—8 days following sectioning of the original nerve In muscles with nerve implants and intact normal innervation hypertrophy occurred amounting to 20—30 per cent within one month

When a foreign motor nerve is surgically implanted into a skeletal muscle synaptic contacts are established provided that the muscle is previously denervated poisoned with botulinum toxin or locally injured A nerve implanted into a normally innervated muscle fails to establish contact with the muscle fibers (Elsberg 1917 Fort 1938, Aitken 1950 Hoffman 1951 Guth and Zalewski 1963 Miledi 1963 Hofmann Thesleff and Zelena 1964)

The purpose of the present investigation was to study the time required for a nerve implant to innervate a denervated rat skeletal muscle Two approaches were used In the first the foreign nerve was implanted simultaneously with the sectioning of the original nerve and in the other series the implant was made 30 days prior to the severance of the normal nerve supply In the latter series the time period between nerve implantation and denervation was designed to allow implanted nerve fibers to grow into the muscle and to mature As reported by Fex and Thesleff (1967) it was to be expected that when the muscle was denervated one month later the implanted nerve fibers would be able to form synaptic contacts within a shorter time period than when implantation and denervation was carried out simultaneously

The hind limb muscles of the rat can be differentiated according to their force velocity properties into fast and slow contracting muscles (Ranvier 1874 Close 1964) These differences in the dynamic properties have been shown to be maintained at least partially, by neural influence Thus, operative cross union of the nerves to fast and slow muscles changes the speed of each muscle so that it is characteristic of the implanted nerve (Buller, Eccles and Eccles 1960) To study the time course of muscle transformation by nerve cross innervation, experiments were made in which a nerve to a fast muscle was implanted into a slow muscle and *vice versa* As previously, two series of rats were used, one in which the nerve was implanted simultaneously with denervation and one with a 30 day interval between implantation and denervation

Following the operative procedures the extent of muscle innervation by each nerve implant was determined by recording isometric twitch tension in response to nerve stimulation and by histochemical visualization of new end plates The dynamic properties of the muscle were studied by comparing the rate of isometric tension development during repetitive stimulation of either the implanted or the original nerve

Methods

Material

The study was carried out on Sprague Dawley rats of both sexes with an initial body weight of 175—200 g The muscles investigated were the flexor digitorum longum (FDL) and the soleus (SOL) The nerves implanted were the tibial nerve (TIBn) and the nerve to FDL (FDLn) both of which are considered to innervate mainly fast-contracting muscles and the nerve to SOL (SOLn) which innervates a slow-contracting muscle The rats were divided into three main groups one in which the TIBn was implanted into FDL a second in which the FDLn was implanted into SOL and a third in which the SOLn was implanted into FDL The nerve implants were made into muscles of the left leg while those of the right served as controls In each main group of rats the original nerve supply in one half of the animals was cut at the time of nerve implantation while in the others it was left intact for an additional period of 30 days at which time severence of the original nerve was carried out in a second operation In this way six series of animals with nerve implants were obtained three series in which denervation was made at the time of nerve implantation and three series with intact original nerve supply for 30 more days Subsequently each series of the animals was divided into six subgroups and 2 4 8 16 and 32 days after denervation the dynamic properties of the muscles were examined In addition the muscles in one subgroup in each series with simultaneous implantation and denervation were examined 62 days afterwards In those with a 30 days interval between implantation and denervation one subgroup was examined at the day of denervation

Anesthesia and surgical procedure

The rats were anesthetized by intraperitoneal administration of pentobarbital sodium 40 mg/kg which during surgery was supplemented with light ether inhalation anesthesia When muscle tensions were recorded however no ether was used For nerve implantation into SOL the lateral and proximal part of the muscle was exposed The nerve innervating FDL was cut close to its muscle and dissected sufficiently free to be brought to the surface of SOL through a small (1 mm or less cut in the fascia covering this muscle The nerve was glued to the surface of SOL by the application of fibrinogen and thrombin and one drop of Ringer's solution Extreme care was taken not to further damage neither the nerve nor the muscle and it is considered unlikely that more than 20—30 muscle fibers were injured by the operative procedure To avoid interference with blood circulation to the muscle animals in which bleeding occurred in the region of the implant were discarded The original nerve supply was cut close to the muscle the proximal nerve stump dissected free ligated and sutured into a deep cut in an adjacent muscle At the day of the final recording a check was made that reinnervation had not occurred

When the TIBn or the SOLn were implanted into the FDL, the technique was the same as that described for SOL It should however, be mentioned that, of these nerves the one innervating SOL is short and for that reason considerable difficulties were encountered when implants were made into the FDI To reach the FDL the SOLn had to be brought through a thick and tight fascia through which the underlying muscle had a tendency to protrude after the incision In the FDL, nerve implants were made in the middle of the muscle, i e close to the original end plate zone This site of implantation had to be chosen because of the shortness of the SOLn

Registration of muscle tension

The muscles to be studied were dissected free in their distal part, the blood supply being preserved Individual motor nerves were carefully dissected, cut centrally and mounted on bipolar platinum stimulating electrodes The muscle tendon was firmly tied to a steel hook which was directly attached to the strain gauge (Grass FT 03) The leg was clamped rigidly at two points, through the foot and through a steel twist drill inserted through the distal part of the femur Muscle and nerves were kept moist by superfusing them with Ringer's solution bubbled with 5 % carbon dioxide in oxygen The composition of the Ringer's solution was that described by Liley (1956) The temperature of the leg was maintained between 36 0—37 0° C by the use of a thermistor and heating lamp

Nerves were stimulated by two Grass S4 stimulators, one modulating the other, and all pulses were fed to the nerve through a Grass stimulus isolation unit Unless otherwise stated each muscle was stimulated at the frequencies of 1, 10 15, 20, 30, 45, 90, 120 and 200 per sec with a square pulse of 0 1 msec duration and supramaximal voltage (8—10 V) The duration of each train of stimuli was 0 4 sec and the interval between them 3 min The stimuli were monitored on an oscilloscope and calibrated with the help of a count down circuit driven from a 10 KC/sec quartz crystal

The strain gauge was aligned in the natural line of pull of the muscle and the initial tension adjusted to give a maximal twitch response as described by Close (1964) The position and initial tension of the strain gauge was repeatedly checked during the experiment The output of the strain gauge was displayed on one beam of the oscilloscope (Tektronix 502) and photographed In addition recordings were made on a polygraph (Mingograph 81 having a flat frequency response up to 500 c/s) The strain gauge was calibrated by weights at the beginning and end of each experiment

The following definitions were used for expressing the mechanical parameters of the muscles studied twitch tension as the maximal mechanical tension produced by a single twitch tetanic tension as the maximum tension reached during the tetanic train fusion frequency as the stimulus frequency at which no apparent oscillations of the tension record were seen at the amplification used contraction time as the time taken for the tension of a single twitch to reach from zero to its peak value (Tc) and relaxation time as time for tension to decline to 50 % of its maximal value (T 1/2 R)

Morphology

At the conclusion of an experiment the muscles used and the control muscles from the contralateral leg were dissected out of the body blotted "dry with filter paper and weighed Similarly the body weights of the rats were noted

The soleus muscle was chosen for a subsequent histological study The growth of implanted nerve fibers into the muscle was observed in frozen longitudinal sections 30 μ thick stained according to Gros-Bielschowski following fixation in AFA solution and 20 % formol

To investigate the extent of formation of new end plates by the nerve implant a histochemical technique for visualizing cholinesterase activity was used The muscles were fixed in 10 % formaldehyde solution at 4 C for a period of 2 or 4 hrs Longitudinal serial sections 60 μ thick were cut from the frozen muscle and stained for cholinesterase according to Gerebtzoff (1953) a modification of the technique of Koelle and Friedenwald (1949) The sections were incubated at 37° C for 1 2 and 10 or 15 hrs at pH 5 with acetylthiocholine iodide as substrate The short incubation time was used for visualizing structural details while the longer incubation times were used for estimation of the new zone of motor end plates All cholinesterase positive loci were checked under high magnification (400 ×) in order to ascertain that they [illegible]

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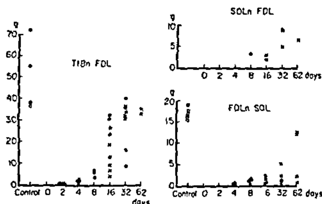


Fig. 1. Isometric twitch tensions following stimulation of respective nerve implants in the FDL and SOL muscles. The open circles are the values obtained in normal muscles stimulated through their original nerve supply. The filled circles are the values obtained from individual muscles in the series with implantation made 30 days prior to denervation and the crosses the values of the series with simultaneous implantation and denervation. Abscissae: days following section of the original nerve supply.

Results

Isometric contractions of muscles innervated by nerve implants

Two approaches were used for studying reinnervation of muscles by nerve implants. In one, surgical implantation and denervation of the original nerve supply to the muscle were carried out in the same operation. In the other series of animals nerve implantation was made into the innervated muscle and 30 days later the original nerve was severed.

Twitch tension. The tensions observed in the two series of rats at various time periods following denervation are given in Fig. 1.

In the series with implantation made prior to denervation *synaptic contacts were* formed 2—4 days after sectioning of the original nerve and subsequently their number increased as shown by a progressively increasing twitch tension. After one month, the mean twitch tension in the FDL upon stimulation of the implanted TIBn was more than 30 g, *i.e.* about the same as that recorded in a normal muscle (see Fig. 1). With implantation of the SOLn, the mean tension of FDL after one month was only 8.9 g. The difference in the extent of innervation of FDL by the two nerves may reflect the functional difference between the nerves but could also be explained by the shortness of the SOLn which made its surgical implantation difficult. For instance, the small number of animals in the two series innervated by SOLn is a reflection of the fact that the nerve, in a majority of the rats, was torn away from its site of attachment and thus failed to innervate the muscle. Stimulation of the FDLn, which normally innervates fast contracting muscles, after implantation into SOL, in both series, produced weak twitches equal to about one tenth that of the normal SOL.

In two groups of rats, stimulation of the implanted nerve produced, even before denervation, a weak twitch response (<50 mg). Since an injured muscle fiber is known to accept innervation by a nerve implant, even though its original nerve supply is intact (Miledi 1963), *the observed twitch is likely to be the result of innervation of damaged muscle fibers* at the site of nerve implantation.

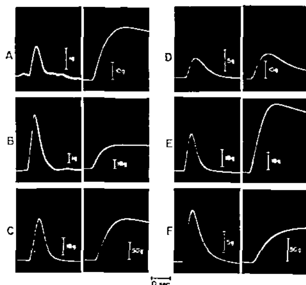


Fig 2 Records of single isometric twitches and tetanic tensions (200 imp/sec) in FDL and SOL following stimulation of the implanted TIBn (records A B and C) and FDLn (records D E and F) respectively The TIBn was implanted into FDL 30 days prior to section of its original nerve and the records obtained 4 days (A) 8 days (B) and 16 days (C) after denervation The FDLn was implanted into SOL simultaneously with denervation of the muscle and the tensions recorded in records D E and F were obtained respectively 16 32 and 62 days later

In contrast to the previous series the rats in which implantation and denervation were made simultaneously in the FDL or in the SOL required 16 days for the formation of functioning synapses (Fig 1) However after 30—60 days the twitch tension was similar to that observed in the series with a 30 days interval between implantation and denervation

Twitch tetanus ratio When the tetanic tension at apparent fusion frequency was examined it was obvious that the tension was not always maintained for the period of 0 4 sec that the impulse train lasted If a fall of tension by more than 10 % of maximal tension occurred it was considered that tension was not maintained This fact made estimations of twitch tetanus ratios uncertain Particularly in SOL it was notable that in the series with a 30 days interval between implantation and denervation tetanic tension was maintained in the early groups (4—8 days) and in the late group (32 days) but not in the groups of 16 days In the series with simultaneous implantation and denervation tetanic tension was maintained only in the late groups Some of these differences are illustrated in Fig 2 which shows typical records of single twitches and tetanic tension in the two series at varying time intervals following denervation

Contraction speed and fusion frequency Nerve implants were made into fast and slow muscles so that the motoneurones (FDLn) which normally innervated fast muscles came to innervate the slow muscle (SOL) and *vice versa* motoneurones of SOLn were made to innervate FDL Additionally the TIBn innervating fast muscles was implanted into the fast FDL From the studies of Buller Eccles and Eccles (1960), Buller and Lewis 1965 and Close 1965) it is known that such cross-innervations affect the speed of muscle contraction by changing the time course of

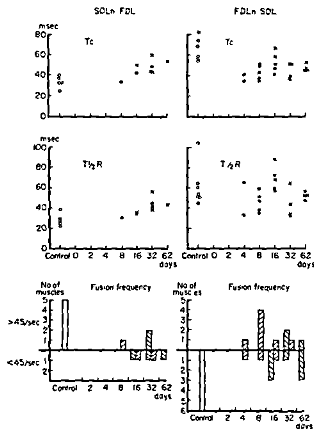


Fig 3 A graphic presentation of the speed of contraction (Tc) the relaxation time (T 1/2 R) and fusion frequency of the FDL and SOL following cross innervation The open circles and ☐ represent the values of normal muscles stimulated through their original nerve supply The filled circles and ☐ are the results obtained in series with nerve implants made 30 days prior to denervation and the crosses and ☐ the values of the series with simultaneous implantation and denervation Abscissae denote the days following section of the original nerve

the rising and falling phases of the twitch contraction and also the summation of tetanic contractions at various frequencies These dynamic properties of the muscle were therefore determined in the 6 series of rats at varying time intervals after denervation the results being presented in Fig 3

Compared with the control values for the normal FDL and SOL it is obvious that in the series of rats studied there was neither a uniform nor a consistent change in the dynamic properties following cross innervation However despite the small number of animals in some of the groups it appeared that implantation of FDLn into SOL modified the contraction time (Tc) half relaxation time (T 1/2 R), and the fusion frequency of the muscle so that it resembled a fast muscle This change was apparent at four and eight days after denervation in the groups with a 30 days interval between implantation and denervation A similar tendency to a modification of the dynamic properties of the muscle in accordance with the innervating motoneurone was also observed in the series with implantation of SOLn into FDL These changes are illustrated in Fig 4 which shows responses of representative FDL and SOL muscles with nerve implants of respectively SOLn and FDLn It is however, noteworthy that implantation of the fast TIBn into the fast FDL also

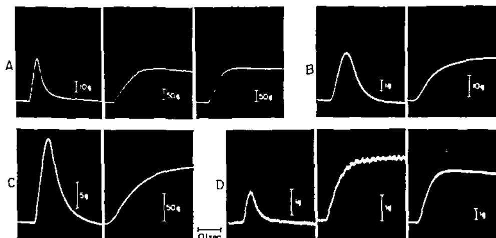


Fig 4 Records showing the contraction and relaxation speed of single twitches and the amount of fusion at a stimulation frequency of 45/sec or 90/sec Record A is from a normal FDL and shows that the apparent fusion frequency is higher than 45/sec Record B was obtained 32 days after implantation of SOLn into FDL and simultaneous denervation Note the

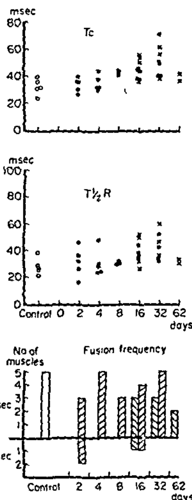
seemed to slow the contraction speed of the muscle (Fig 5) This observation is in agreement with Eccles Eccles and Kozak (1962) who pointed out that the twitches of fast muscles were greatly slowed by denervation while with slow muscles the slowing is much less

Morphology

The original zone of end plates and the growth of implanted nerve fibers

The original end plates when stained for cholinesterase activity appear as a characteristic zig zag pattern approximately in the central region of each muscle The characteristic localization of those motor end plates which retained their cholinesterase activity for a long period after denervation helped to distinguish original end plates from the new junctions established during the experiment Additionally after denervation the sub neural apparatus of original end plates exhibit shrinkage and granulation (Csillik 1959)

Utilizing silver impregnation the terminals of the implanted nerve were found in longitudinal sections of the muscle 30 days after implantation but before section of the original nerve supply the implanted nerve stump and its nerve terminals were no more than 1—2 mm from the site of implantation The original motor nerve terminals on the other hand were localized in the middle part of the muscle The fibers of the implanted nerve were observed to run in parallel to muscle fibers or in a plexiform network but structures corresponding to end plates were very rare In



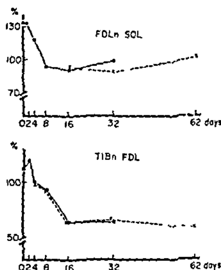


Fig 5 Fig 6

Fig 5 Same as Fig 3 but illustrates the effects of implanting the TIBn into FDI

Fig 6 A graphic presentation of the wet weights of the SOL and FDI muscles with nerve implants of respectively FDLn and TIBn The values are the means of the muscles presented in the graphs of Fig 1 and are expressed in percent of respective contralateral muscle The filled circles are those with 30 days pre implantation and the crosses from the series with simultaneous implantation and denervation Note that in both muscles nerve implants without denervation caused an apparent increase in muscle weights

such structures in comparison with normal end plates (Fig 8 4) usually one or at the most a few branches were observed and the number of the nuclei as far as they were visible were reduced (Fig 8 5 6)

New end-plates In SOL muscles in which nerve implants were made one month before denervation at the day of sectioning of the original nerve a few new end plates were observed in the immediate vicinity of the implantation site Two days after severing the original nerve the number of new end plates was still small (20 – 30) but 4 days after denervation the number of new junctions was almost twice that



Fig 7 Histogram of muscle fibre diameters from the control (C white columns) and experimental (E black columns) soleus muscles one month after the nerve implantation into the innervated muscle Abscissa distribution of muscles fibre diameters in 5μ intervals. Ordinate the number of muscle fibers in each group

amount A more substantial increment in the number of newly formed junctions was found in muscles 8 days after denervation, when in 2 muscles out of 4, there were almost 200 end plates At that period there was a distinct shift of the new end plate zone distally so that some end plates were found 1/3 of the distance between the site of implantation and the original end plate zone

At later stages of the experiment *i e* 16 and 32 days after section of the original nerve the number of end plates gradually increased and the size of the new zone became larger 16 days after section of the original nerve (Fig 9 1—5) numerous fibers of the implanted nerve had grown out in various directions from the site of implantation and established end plates up to about 2/3 of the distance between the implantation site and the original motor end plate zone

As seen in serial sections the region containing new end plates was usually spread out in superficial layers of the muscle (Fig 9 1—3) while in deeper layers it was more circumscribed and the end plates were less numerous (Fig 9 4 5)

In muscles with simultaneous nerve implantation and denervation end plates formed by the implanted nerve were present from the 16th day after the operation The newly formed zone was at that time localized to the vicinity of the site of implantation and the number of motor end plates was substantially lower than in muscles with a pre implanted nerve at the same time interval (*i e* 16 days after section of the original nerve) it was also lower than in muscles with a pre implanted nerve at 8 days denervation At later periods *i e* 32 and 62 days after the operation the number of end plates and the size of the newly formed end plate zone gradually increased approaching the range of values found in the pre implanted muscle

Some of the original end plates may have become reinnervated by the implanted nerve during the experiment however most of them exhibited granulation reduced cholinesterase activity and a diffuse staining typical for denervated end plates (Gullik 1959)

Staining for cholinesterase activity visualizes the post synaptic membrane of the end plate In a normal muscle the end plates have a regular oval or round shape formed by the interconnected synaptic troughs (Fig 8 1) The size and shape of

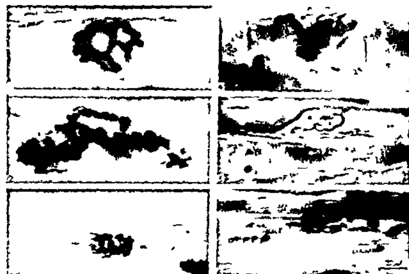


Fig. 8. Original end plates in the soleus muscle (1 and 4) and newly formed end plates in the soleus after nerve implantation (2, 3, 5 and 6). 1—3: cholinesterase staining; 4—6: silver impregnation. Scale 50 μ. Incubation time for cholinesterase staining: 1 hr.

newly formed end plates varied (Fig. 8: 2, 3), especially in muscles studied 16 and 32 days after denervation. In most of the new end plates the post-synaptic membrane consisted of small separate lobes which either accumulated in great number round the nerve terminals (Fig. 8: 2) or as a few arranged in a group (Fig. 8: 3) (Koenig 1963; Fex et al. 1966).

Muscle weight and fiber diameter. Since denervation produces atrophy of muscle fibers, it was of interest to determine the weight of the muscles in the two series. In addition, in a few instances, the average transverse fiber diameters were measured.

The graphs in Fig. 6 illustrate the muscle weights expressed as a percentage of those of the contralateral control muscle. Denervation produced in FDL and SOL a fall of weight, the weight loss being most marked in the FDL. Recovery of weight occurred in the SOL while the weight of the FDL remained at a level of about 60 per cent of normal for the entire period of observation.

Normally innervated muscles with nerve implants showed hypertrophy at the time the original nerve was sectioned, the gain in weight corresponding to 1–33 per cent of the weight of the contralateral muscles. The increase in weight was maintained during the first days after cutting the original nerve, but by the 8th day a decrease of muscle weight was apparent. The hypertrophy was accompanied by an increase in muscle fiber diameters as shown for SOL in the histogram of Fig. 7. In the group of 6 rats with normal innervation 30 days after implantation of the FDLn, the mean fiber diameter in the soleus muscle was 41.1 ± 0.65 μ (S.E. of mean), whereas the mean fiber diameter in contralateral muscles was 33.1 ± 0.79 μ.



Fig 9 Longitudinal sections through soleus muscle 46 days after nerve pre implantation and 16 days after section of the original nerve 1—3 superficial sections 4—5 sections of deeper regions. Note new end plate zone around the implanted nerve at the top and original end plate region in the middle of the sections Cholinesterase staining scale 1 mm Incubation time 10 hrs

Discussion

In the present study two kinds of nerve implants were made One in which the nerve was implanted into a normally innervated muscle and one in which the implant was made simultaneously with section of the original nerve It was expected that, with the first mentioned procedure the fibers of the implanted nerve would grow into the muscle and form mature nerve endings When the original nerve was sectioned 30 days later and the muscle thereby denervated the implanted nerve terminals should be able to rapidly form extensive synaptic contacts without further growth In the second series with concomitant implantation and denervation the implanted nerve fibers would have to grow into the muscle before functional contacts could be established resulting presumably in a slower and more gradual reinnervation of the muscle

The experimental results only partly confirmed these expectations Muscle twitches in response to stimulation of the implanted nerve were obtained within one week after denervation in the series with an interval of 30 days between nerve implantation and denervation This was in contrast to the rats in which the two procedures were made in one operation In those rats about two weeks were required to establish functioning neuromuscular contacts

In the muscles with one month of pre implantation denervation resulted in functional innervation of a considerable number of muscle fibers within 8 days as judged by the force of muscle contraction in response to stimulation of the nerve implant. The number of new end plates as visualized by staining for cholinesterase activity

however, appeared to be relatively small. This suggests that the process of forming functioning synaptic contacts occurs more rapidly than the induction of cholinesterase activity. A gradual increase in the extent of reinnervation by the implanted nerve at later stages and the shift of the newly formed end plate zone towards the center of the muscle demonstrated that the growth of nerve fibers and end plate formation were progressing. Why this occurred gradually, during a relatively long time period instead of within a short period following denervation is unclear.

The twitch tensions presented in Fig. 1 show that when TIBn, which mainly innervates fast muscles, is implanted into FDL a more extensive innervation is achieved than when the SOLn is used. This difference could imply that a 'fast' nerve more readily forms functional contacts with a fast muscle than does a 'slow' nerve, but it could also be a reflection of a difference in the number of axons in the two nerve trunks, the TIBn being considerable larger than the SOLn.

Buller, Eccles and Eccles (1960), Eccles *et al.* (1962), Buller and Lewis (1965) and Close (1965) have reported changes in the dynamic characteristics of slow and fast muscles following their cross innervation. In all those studies the original nerve to the muscle was cut at the time of nerve implantation and the force velocity properties of the muscles studied not earlier than 28 days after the operation. Thus no data were available about the early changes in the rate of isometric tension development in mammalian fast and slow muscles following operative cross implantation of their respective nerves. From our data as presented in Fig. 3 it seems that implantation of a fast nerve (FDLn) into SOL modified the speeds of muscle contraction and relaxation towards those of a fast muscle and that a similar but less marked change in the opposite direction occurred when SOLn was implanted into FDL. It is of interest that these changes were observed as early as 4—8 days after section of the original nerve indicating that the nerve factors which determine the speed of the muscle assert themselves at an early phase of innervation.

Hypertrophy by about 10 per cent has previously been shown to occur in normally innervated muscles into which a foreign nerve was implanted (Guth and Zalewski 1963) and has also been found to a similar extent in muscles with a true accessory nerve supply (Gutmann and Hanzlíková 1967). In our experiments 30 days after nerve implantation only occasional newly formed end plates were observed and therefore accessory innervation with hyperfunction can be excluded as the cause of the 30 per cent relative hypertrophy observed. The increase in the muscle weights and in the muscle fiber diameter in the SOL suggest that the free nerve terminals of the implanted nerve by some mechanism have exerted a trophic influence on the muscle resulting in its hypertrophy. However, such a hypothesis attractive as it may be, is still premature as we do not know the dry weight values of the muscles and therefore cannot exclude that at least a part of the hypertrophy was due to some pathological reaction of the muscle to the nerve implant.

The study was supported by a research grant from the Swedish Medical Research Council (14X-732-03), Stockholm and by grants from the Medical Faculty, University [illegible] Sweden

References

AITKEN, J T, Growth of nerve implants in voluntary muscle *J Anat* (Lond) 1950 *84* 38—49

BULLER A J, J C ECCLES and ROSAMOND ECCLES, Interactions between motoneurones and muscles in respect of the characteristic speeds of their responses *J Physiol* (Lond) 1960 *150* 417—439

BULLER A J and D M LEWIS, Further observations on mammalian cross innervated skeletal muscle *J Physiol* (Lond) 1965 *178* 343—358

C ... development

C ... uscles *Nature*

CSILLIK, B, *Functional structure of the postsynaptic membrane in the myoneural junction* Publ House Hung Acad Sci 1959 Budapest 1965

ECCLES, J C, ROSAMOND M ECCLES and W KOZAK, Further investigations on the influence of motoneurones on the speed of muscle contraction *J Physiol* (Lond) 1962 *163* 324—339

ELSBERG C A, Experiments in motor ... lyzed muscles by their own and by ...

FEX, S, B SONESSON, S THESLEFF ... mammalian muscle *J Physiol* (Lc

FEX, S and S THESLEFF, The time required for innervation of denervated muscles by nerve implants *Life Sciences* 1967 *6* 635—639

FORT, W B, *An experimental study of the factors involved in the establishment of neuromuscular connections* Dissertation, Dept Biol Univ of Chicago 1938 1—88

GEREBTZOFF, M A Recherches histochimiques sur les acétylcholine et choline esterase *Acta anat* (Basel) 1953 *19* 366—379

GUTH L and A A ZALEWSKI, Disposition of cholinesterase following implantation of nerve into innervated and denervated muscle *Exp Neurol* 1963 *7* 316—326

GUTMANN, E and V HANZLÍKOVÁ Effects of accessory nerve supply to muscle achieved by implantation into muscle during regeneration of its nerve *Physiol bohemoslov* 1967 *16* 244—250

HOFFMAN H, A study of the factors influencing innervation of muscles by implanted nerves *Austr J exp Biol Med Science* 1951 XXIX 289—308

HOFMANN, W W, S THESLEFF and J ZELENA, Innervation of botulinum poisoned skeletal muscles by accessory nerves *J Physiol* (Lond) 1964 *171* 27—28P

KOELLE, G B and J S FRIEDENWALD, A histochemical method for localizing cholinesterase activity *Proc Soc exp Biol* (N Y) 1949 *70* 617—622

KOENIG, J, Innervation motrice expérimentale d une portion de muscle strie normalement depourvue de plaque motrices chez le Rat *C r hebd Seanc Acad Sci* Paris 1963 *256* 2918—2920

LILEY, A W An investigation of spontaneous activity at the neuromuscular junction of the rat *J Physiol* (Lond) 1956 *132* 650—666

MILEDI, R, Formation of extra nerve muscle junctions on innervated muscle *Nature* (Lond) 1963 *199* 1191—1192

RANVIER, L, De quelques faits relatifs à l histologie et a la physiologie des muscles stries *Arch Physiol norm path* 1874 *6* 1—15

fusion with Na free Krebs Henseleit solution either phenoxybenzamine (PBA, Dibenzyline® Smith, Kline and French) 10 $\mu g/ml$ or cocaine 8 $\mu g/ml$ + Hydergin® (Sandoz) 0.3 $\mu g/ml$ were added to the perfusion medium. After 10 min the spleen was divided into two portions one to serve as a control and the other to be used for the nerve stimulations. The division was made after applying a tightly fitting clamp, in the mostly easily observable borderline between the two vascular beds of the spleen. The control portion was immediately blotted dry, weighed and homogenized in an Ultra-Turrax apparatus with 10 volumes of 10 % trichloroacetic acid. Electrical nerve stimulations of the remaining portion of the spleen were carried out as described above. At the end of the experiment the stimulated portion of the spleen was weighed, homogenized and extracted in the same way as the control.

Analysis

The outflow of NA from the spleen was monitored by determining the radioactivity in the different crude perfusate fractions in a Packard Liquid Spectrometer, as described below.

The remainder of the perfusate fractions was then pooled in groups, and protein was precipitated by addition of 1/10 volume of 50 % trichloroacetic acid, or in the samples used for chromatography by addition of 1/10 volume of 4 M perchloric acid.

The perfusate samples and the organ extracts were purified on alumina and their NA content assayed fluorimetrically according to Euler and Lishajko (1961). In order to determine NA metabolites, aliquots of representative perfusate samples and organ extracts were also analysed by cation exchange column chromatography as described by Stjärne and Lishajko (1967).

The radioactivity of the different samples was determined by counting 0.5 ml aliquots in a 7 : 3 toluene absolute ethanol solution containing 4 g of 2, 5 diphenyloxazole and 100 mg of 1-4 bis 2(4 methyl 5 phenyloxazolyl) benzene per liter of toluene. Quenching was monitored by internal standards of ^{3}H dl NA and was found not to differ significantly between different extracts.

According to chromatographic analysis of the perfusate fraction obtained during the period of maximum NA overflow in response to nerve stimulation NA accounted for 96.3 % ± 0.9 (mean ± SEM, n = 4) of the total radioactivity, and in the pooled total perfusate for 90.6 % ± 0.6 (mean ± SEM, n = 5). In the perfused spleen 96.2 % ± 1.0 (mean ± SEM, n = 1) of the radioactivity consisted of intact NA.

In control experiments the recovery of NA added to perfusate samples and to organ extracts and carried through the entire procedure of purification and fluorimetrical determination was 75.9 % ± 1.3 (mean ± SEM, n = 15) in the perfusate and 78.1 % ± 1.2 (mean ± SEM, n = 15) in the splenic extracts.

The NA values in splenic extracts obtained by fluorimetric assay were corrected for losses on alumina (correction factor 1/0.781), and those in the perfusate both for losses on alumina and for metabolites (correction factor for NA peak 1/0.759 × 0.963, and for total perfusate 1/0.759 × 0.906).

The initial NA content of the stimulated portion of the spleen was calculated from the NA content of the control portion of the spleen, using the deoxyribonucleic acid (DNA) content of the two portions of the spleen as a reference (cf. Dearnaley and Geffen 1966) in the following way.

The acid insoluble sediment remaining after centrifugation of the splenic homogenate was treated with 0.5 M perchloric acid at 80° C for 20 min. Purine deoxyribose in the acid phase was then determined colorimetrically by the diphenylamine reaction according to Burton (1956). The results were expressed in DNA units, 1 unit representing the extinction caused by 1 mg of standard DNA (deoxyribonucleic acid sperm, National Biochem. Corp.) treated in the same way as the tissue sediment.

Using this method the NA content of the [illegible] of a series of non stimulated cat spleens was found to [illegible] al (Table [illegible]

Thus by determinat[illegible] ntent of [illegible] rtion and of the DNA content of both the control ar[illegible] rtions [illegible] to calculate the NA content of the stimulated port[illegible] the [illegible] the organ.

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TABLE I Distribution of NA in control cat spleens in relation to wet weight (w w) and to DNA

	A w w	B DNA units	C μg NA	C/A	C/B	B/A
1	3.15	27.5	6.92	2.20	0.252	8.73
	3.95	35.5	8.71	2.21	0.245	8.99
2	2.15	16.0	0.75	1.74	0.234	7.44
	1.80	14.0	3.22	1.79	0.230	7.77
3	2.20	17.0	3.39	1.54	0.199	7.73
	2.70	20.3	3.89	1.44	0.192	7.52
4	2.65	20.0	3.57	1.35	0.179	7.55
	3.30	27.0	4.85	1.47	0.180	8.18
5	1.40	12.3	2.07	1.48	0.168	8.79
	1.30	11.3	1.85	1.42	0.164	8.69
	1.75	16.8	3.70	2.11	0.220	9.60
	3.75	34.0	7.82	2.09	0.230	9.10

TABLE II Outflow of NA (ng/g w w /min uncorrected) from isolated perfused cat spleen after pretreatment with nialamide (100 mg/kg i v to the animal) and addition to the perfusion fluid of PBA (10 μg/ml) or cocaine (8 μg/ml) + Hydergin (0.3 μg/ml.) Mean values ±SEM

Drugs	Control	Nialamide	Nialamide PBA	Nialamide Cocaine Hydergine
NA ng g w w /min	0.53 ± 0.11	0.56 ± 0.06	0.59 + 0.08	0.58 ± 0.03
Number of experiments	9	7	5	4

Hydergin did not significantly alter the fluorimetrically determined basal NA overflow (Table II) or the level of radioactivity in the perfusate found in the absence of these drugs (Fig 1 and 2) Nor did pretreatment with nialamide change the normal NA content of the spleen (Table III)

When PBA or cocaine + Hydergin were added to the perfusion fluid electrical stimulation of the splenic nerves resulted in an immediate large rise in the outflow of NA At a stimulation frequency of 1/sec the peak NA outflow was 4.19 + 0.68 ng/DNA unit/min (mean + SEM n = 4) When higher stimulation frequencies were used (5/sec and 10/sec) the peak outflow was always higher At 5/sec the NA outflow rose to 14.68 ± 2.10 ng/DNA/min and at 10/sec to 12.19 ± 3.60 ng/DNA unit/min (mean ± SEM n = 4) (cf Table IV)

fusion with NA free Krebs Henseleit solution either phenoxybenzamine (PBA[1] Dibenzline[®] Smith, Kline and French) 10 μg/ml, or cocaine 8 μg/ml + Hydergin[®] (Sandoz) 0.3 μg/ml were added to the perfusion medium. After 10 min the spleen was divided into two portions one to serve as a control and the other to be used for the nerve stimulations. The division was made, after applying a tightly fitting clamp in the mostly easily observable borderline between the two vascular beds of the spleen. The control portion was immediately blotted dry, weighed and homogenized in an Ultra Turrax apparatus with 10 volumes of 10 % trichloroacetic acid. Electrical nerve stimulations of the remaining portion of the spleen were carried out as described above. At the end of the experiment the stimulated portion of the spleen was weighed, homogenized and extracted in the same way as the control.

Analysis

The outflow of NA from the spleen was monitored by determining the radioactivity in the different crude perfusate fractions in a Packard Liquid Spectrometer as described below.

The remainder of the perfusate fractions was then pooled in groups and protein was precipitated by addition of 1/10 volume of 50 % trichloroacetic acid or in the samples used for chromatography by addition of 1/10 volume of 4 M perchloric acid.

The perfusate samples and the organ extracts were purified on alumina and their NA content assayed fluorimetrically according to Euler and Lishajko (1961). In order to determine NA metabolites aliquots of representative perfusate samples and organ extracts were also analysed by cation exchange column chromatography as described by Stjärne and Lishajko (1967).

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Using this method the NA content of the two portions of a series of non-stimulated cat spleens was found to be almost identical (Table I).

Thus by determination of the NA content of the control portion and of the DNA content of both the control and the stimulated portions it was possible to calculate the NA content of the stimulated portion of the spleen at the time of division of the organ.

Results

In the absence of nerve activity the NA level in the effluent from the spleen was quite low and close to the limit of satisfactory accuracy in the fluorimetric NA determination with the method used. The values found indicate that pretreatment of the cats with nialamide or addition to the perfusion fluid of PBA or cocaine +

TABLE I Distribution of NA in control cat spleens in relation to wet weight (w w) and to DNA

	A w w	B DNA units	C µg NA	C/A	C/B	B/A
1	3 15	27 5	6 92	2 20	0 252	8 73
	3 95	35 5	8 71	2 21	0 245	8 99
2	2 15	16 0	0 75	1 74	0 234	7 44
	1 80	14 0	3 22	1 79	0 230	7 77
3	2 20	17 0	3 39	1 54	0 199	7 73
	2 70	20 3	3 89	1 44	0 192	7 52
4	2 65	20 0	3 57	1 35	0 179	7 55
	3 30	27 0	4 85	1 47	0 180	8 18
5	1 40	12 3	2 07	1 48	0 168	8 79
	1 30	11 3	1 85	1 42	0 164	8 69
	1 75	16 8	3 70	2 11	0 220	9 60
	3 75	34 0	7 82	2 09	0 230	9 10

TABLE II Outflow of NA (ng/g w w /min uncorrected) from isolated perfused cat spleen after pretreatment with nialamide (100 mg/kg i v to the animal) and addition to the perfusion fluid of PBA (10 µg/ml) or cocaine (8 µg/ml) + Hydergin (0 3 µg/ml) Mean values ±SEM

Drugs	Control	Nialamide	Nialamide PBA	Nialamide Cocaine Hydergine
NA ng/g w w /min	0 53±0 11	0 56±0 06	0 59±0 08	0 58±0 03
Number of experiments	9	7	5	4

Hydergin did not significantly alter the fluorimetrically determined basal NA overflow (Table II) or the level of radioactivity in the perfusate found in the absence of these drugs (Fig 1 and 2) Nor did pretreatment with nialamide change the normal NA content of the spleen (Table III)

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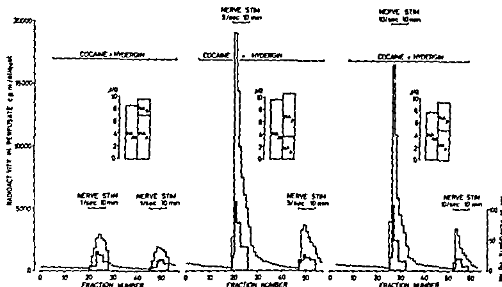


Fig 1 Perfused cat spleens pretreated with malamide and loaded with ³H dl NA Outflo of radioactivity and of fluorimetrically determined NA (shaded areas) from the spleen resting and in response to nerve stimulation Cocaine + hydergin added to the perfusion medium to inhibit uptake and to facilitate washout of NA released from the sympathetic neurons Calculated starting NA content in stimulated spleen (NA_s) NA content in stimulated spleen at the end of the experiment (NA_e) and NA content in perfusate (NA_f)

At 1/sec the peak level of NA overflow was relatively well maintained throughout the stimulation period or decreased only moderately With the higher stimulation frequencies the NA outflow reached a higher peak level only to fall quite steeply (Fig 1 2)

The peak level of the NA overflow response to a second nerve stimulation period after a resting interval of 20—30 min was always lower than the peak level in the first stimulation period and closer to the NA level towards the end of the first stimulation period (Fig 1, 2)

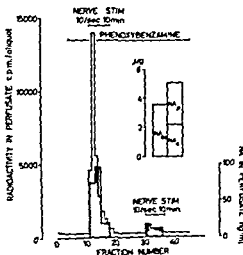


Fig 2 Perfused cat spleen pretreated with nialamide and loaded with ³H dl NA Outflow of radioactivity and of fluorimetrically determined NA shaded areas from the spleen resting and in response to nerve stimulation PPA added to the perfusion medium to inhibit uptake and to facilitate washout of NA released from the sympathetic neurons Calculated starting NA content in stimulated spleen NA_s NA content in stimulated spleen at the end of the experiment NA_e and NA content in perfusate NA_f

TABLE III Effect of MAO inhibition on NA content in cat spleen Nialamide 100 mg/kg given 1—2 hours before the experiment Mean values ± SEM

Number of experiments	Drug treatment	NA μg/g w w	NA μg/DNA-unit
6	0	1 74 ± 0 14	0 21 ± 0 01
15	nialamide	1 78 ± 0 13	0 20 ± 0 02

TABLE IV NA synthesis in, and maximum overflow from 12 cat spleens in response to nerve stimulation of different frequencies Mean values ± SEM Relation of synthesis to maximal overflow Multiplying synthesis and overflow values by 9 4 gives an estimate of synthesis and overflow of NA expressed in ng/g w w /min

No	Stimulation Frequency	Synthesis ng NA/DNA-U/min		Overflow ng NA/DNA-U/min		Synthesis/overflow %	
1	1/sec	0 35		3 74		9 4	
2		0 18		2 46		8 1	
3		0 85		5 16		16 4	
4		0 40		5 41		7 4	
			0 45 ± 0 14		4 19 + 0 69		10 3 ± 2 1
1	5/sec	0 42		17 20		2 5	
2		0 27		8 61		3 1	
3		0 40		17 90		2 2	
4		0 74		15 10		4 9	
			0 46 ± 0 10		14 68 ± 2 10		3 2 ± 0 6
1	10/sec	0 14		10 47		1 3	
2		0 25		7 69		3 3	
3		0 74		7 79		9 5	
4		0 67		22 80		2 9	
			0 45 ± 0 15		12 19 ± 3 60		4 3 ± 1 8

The total amount of NA recovered in the perfusate from the stimulated portion of the spleen (NA_t) was very large both absolutely and in relation to the calculated starting NA content of the stimulated portion of the spleen (NA_{so}), 53 0 ± 4 3 % of NA_{so}, (mean ± SEM, n = 12) (*cf* Table V) The NA content of the stimulated portion of the spleen found at the end of the experiment (NA_s) was always lower than NA_{so} However while there was thus a substantial reduction of the tissue content of NA the depletion was by no means complete (39 0 ± 4 5 % of NA_{so} mean ± SEM n = 12), (*cf* Table V) Since the depletion was less than NA_p, part of the overflowing NA must have been replaced by newly synthesized neurotransmitter

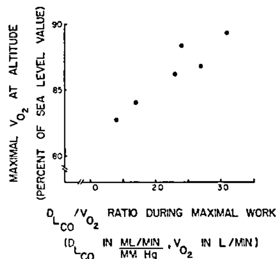


Fig 1 The relationship between reduction in maximal V_{O_2} at 2 250 m a s l and the ratio between the pulmonary diffusing capacity for CO and oxygen uptake at maximal exercise in 6 athletes

Maximal V_{O_2} at sea level was in all 6 subjects slightly lower after than before the stay at altitude The difference was probably related to a reduced intensity of training at altitude Either set of sea level data might have been used to demonstrate the relationship shown in Fig 1 A similar correlation is also present between maximal V_{O_2} at altitude and the ratio $D_{L_{CO}}$/maximal cardiac output at sea level

In young adults a conditioning program induce a larger increase in max V_{O_2} than in $D_{L_{CO}}$ (Saltin et al 1968) This is in accordance with the findings that individuals with a large aerobic work power during maximal exercise have a smaller margin to the upper limit of the lungs to transfer oxygen (Shepard *et al* 1957) The ratio for $D_{L_{CO}}$/max V_{O_2} had a mean value of 12 2 in the present study of endurance athletes compared with 13 in trained students (Holmgren and Åstrand 1966) 14 in untrained students (Saltin *et al* 1968) and 15 in untrained female students (Holmgren and Åstrand 1966) These findings may then explain the observations that the largest reduction in max V_{O_2} at altitude is found in the most well trained groups (Saltin 1967, Grover *et al* 1967, Faulkner *et al* 1968) It is worth noticing however, that is the ratio between $D_{L_{CO}}$ and max V_{O_2}, that is the crucial factor for the reduction in max V_{O_2} at altitude and not an extreemly high max V_{O_2} per see Thus the present findings may also give the physiological explanation for the observed large differences in performance at altitude between athletes of equal capacity at sea level

This study was supported by grants from the Swedish Sports Federation (Pol klinikkommittén) and USPHS (HE 07744 Technical assistance was given by Mrs Inger Hallback and Mr Abraham Prengal

References

FAULKNER J A, J KOLLIAS C B FAVOUR E R BUSKIRK and B BALKE Maximum aerobic capacity and running performance at altitude *J appl Physiol* 1968 *24* 685—691
HOLMGREN A and P O ÅSTRAND D_L and the dimensions and functional capacities of the O_2 transport system in humans *J appl Physiol* 1966 *21* 1463—1470

by local synthesis (Spector, Melmon and Sjoerdsma 1962, Montanari *et al* 1963, Levitt *et al* 1965, Sedvall Weise and Kopin 1968) or by uptake from the environment of preformed NA either circulating (Kopin and Gordon 1963, Kopin, Gordon and Horst 1965) or locally released (Paton 1960, Blakeley, Brown and Geffen 1964, Stjarne 1964, Brown 1965, Gillespie and Kirpekar 1965, Haefely *et al* 1965, Blakeley and Brown 1966 Hedqvist and Stjarne 1966, Geffen 1967, Folkow, Haggendal and Lisander 1968) has been based on studies of either parameter in separate experiments, using different tissues, species and techniques

In view of this the present investigation was carried out to study the balance between the loss of NA from the neuron by spontaneous 'leakage" as well as by neurotransmitter discharge in response to nerve stimulation, and the gain of NA by local synthesis, in one and the same experiment, using the isolated perfused cat spleen Intraaxonal deamination was blocked by inhibition of monoamine oxidase (MAO) O methylation was monitored and corrected for Uptake of NA from the extracellular medium was blocked Thus the corrected values for NA in the venous effluent should equal the total amount of NA given off from the neurons, and the net gain of NA calculated from a balance study should reflect NA synthesis

Abbreviations

NA	=	noradrenaline
MAO	=	monoamine oxidase
PBA	=	phenoxybenzamine
DNA	=	deoxyribonucleic acid
NA_I	=	noradrenaline in perfusate
NA_{so}	=	initial noradrenaline content of stimulated spleen (calculated)
NA_s	–	noradrenaline content of stimulated spleen at the end of the experiment (found)

Methods

Cats of both sexes weighing 2—4 kg were used for the study The experimental animals were anesthetized with sodium pentobarbital (30 mg/kg i p) and pretreated with nialamide (Niamid® Pfizer 100 mg/kg i v about one hour before starting the perfusion of the spleen) and heparin (1000 I U /kg i v The abdomen was opened by a midline incision and the splenic vessels and nerves were car [illegible] then cut The spleen was transferr [illegible] fused with a slightly modified [illegible] $MgSO_4$ 7 H_2O 0 294 g $CaCl_2$ [illegible] KH_2PO_4 0 163 g $NaHCO_3$ 2 1 g dextrose [illegible] containing 2 per cent dextran (Macrodex® Pharmacia M_w 70 000) and aerated with 5 % CO_2 in O_2 The flow rate was kept at [illegible] ml/min by means of a constant rate perfusion pump (Sigmamotor) Ascorbic acid (20 μg/ml was added to the perfusion fluid to protect the NA in the medium and hydrochloric acid was infused into the venous collecting system to maintain a suitable low acidity in the perfusate The effluent from the spleen was divided into 10 ml portions by means of an automatic fraction collector Supramaximal electrical stimulation of the splenic nerves was carried out with shielded platinum electrodes using a Grass S4 stimulator operated at frequencies from 1 10 per sec a duration of 2 msec and a stimulation strength of 20—30 volts for two stimulation periods of 10 20 min duration separated by an interval of 20—30 min

The experiment was started by an intraarterial infusion of 32 μC of tritium labeled dl NA spec act 5 10 mC μmole New England Nuclear Corp After waiting for 30 min by per-

fusion with Na free Krebs Henseleit solution either phenoxybenzamine (PBA[1] Dibenzyline® Smith Kline and French) 10 µg/ml or cocaine 8 µg/ml + Hydergin® (Sandoz) 0.3 µg/ml were added to the perfusion medium. After 10 min the spleen was divided into two portions one to serve as a control and the other to be used for the nerve stimulations. The division was made, after applying a tightly fitting clamp in the mostly easily observable borderline between the two vascular beds of the spleen. The control portion was immediately blotted dry weighed and homogenized in an Ultra Turrax apparatus with 10 volumes of 10 % trichloroacetic acid. Electrical nerve stimulations of the remaining portion of the spleen were carried out as described above. At the end of the experiment the stimulated portion of the spleen was weighed, homogenized and extracted in the same way as the control.

Analysis

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The remainder of the perfusate fractions was then pooled in groups and protein was precipitated by addition of 1/10 volume of 50 % trichloroacetic acid or in the samples used for chromatography by addition of 1/10 volume of 4 M perchloric acid.

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	3.95	35.5	8.71	2.21	0.245	8.99
2	2.15	16.0	0.75	1.74	0.234	7.44
	1.80	14.0	3.22	1.79	0.230	7.77
3	2.20	17.0	3.39	1.54	0.199	7.73
	2.70	20.3	3.89	1.44	0.192	7.52
4	2.65	20.0	3.57	1.35	0.179	7.55
	3.30	27.0	4.85	1.47	0.180	8.18
5	1.40	12.3	2.07	1.48	0.168	8.79
	1.30	11.3	1.85	1.42	0.164	8.69
	1.75	16.8	3.70	2.11	0.220	9.60
	3.75	34.0	7.82	2.09	0.230	9.10

TABLE II Outflow of NA (ng/g w.w./min uncorrected) from isolated perfused cat spleen after pretreatment with nialamide (100 mg/kg i.v. to the animal) and addition to the perfusion fluid of PBA (10 µg/ml) or cocaine (8 µg/ml) + Hydergin (0.3 µg/ml) Mean values ± SEM

Drugs	Control	Nialamide	Nialamide PBA	Nialamide Cocaine Hydergine
NA ng/g w.w./min	0.53 ± 0.11	0.56 ± 0.06	0.59 ± 0.08	0.58 ± 0.03
Number of experiments	9	7	5	4

Hydergin did not significantly alter the fluorimetrically determined basal NA overflow (Table II) or the level of radioactivity in the perfusate found in the absence of these drugs (Fig. 1 and 2). Nor did pretreatment with nialamide change the normal NA content of the spleen (Table III).

When PBA or cocaine + Hydergin were added to the perfusion fluid electrical stimulation of the splenic nerves resulted in an immediate large rise in the outflow of NA. At a stimulation frequency of 1/sec the peak NA outflow was 4.19 ± 0.68 ng/DNA unit/min (mean ± SEM n = 4). When higher stimulation frequencies were used (5/sec and 10/sec) the peak outflow was always higher. At 5/sec the NA outflow rose to 14.68 ± 2.10 ng/DNA/min and at 10/sec to 12.19 ± 3.60 ng/DNA unit/min (mean ± SEM n = 4) (*cf.* Table IV).

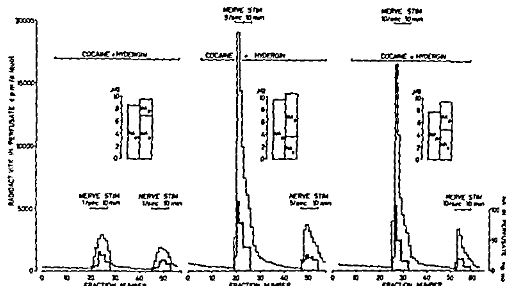


Fig 1 Perfused cat spleens pretreated with nialamide and loaded with ^{3}H dl NA Outflow of radioactivity and of fluorimetrically determined NA (shaded areas) from the spleen resting and in response to nerve stimulation Cocaine+hydergin added to the perfusion medium to inhibit uptake and to facilitate washout of NA released from the sympathetic neurons Calculated starting NA content in stimulated spleen (NA_{s_1}) NA content in stimulated spleen at the end of the experiment (NA_{s_2}) and NA content in perfusate (NA_f)

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The peak level of the NA overflow response to a second nerve stimulation period after a resting interval of 20—30 min was always lower than the peak level in the first stimulation period and closer to the NA level towards the end of the first stimulation period (Fig 1 2)

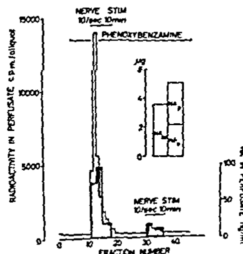


Fig 2 Perfused cat spleen pretreated with nialamide and loaded with ^{3}H-dl NA Outflow of radioactivity and of fluorimetrically determined NA shaded areas from the spleen resting and in response to nerve stimulation PBA added to the perfusion medium to inhibit uptake and to facilitate washout of NA released from the sympathetic neurons Calculated starting NA content in stimulated spleen NA_{s_1} NA content in stimulated spleen at the end of the experiment NA_{s_2} and NA content in perfusate NA_f

TABLE III. Effect of MAO inhibition on NA content in cat spleen. Nialamide 100 mg/kg given 1–2 hours before the experiment. Mean values ± SEM

Number of experiments	Drug treatment	NA μg/g w.w.	NA μg/DNA unit
6	0	1.74 ± 0.14	0.21 ± 0.01
15	nialamide	1.78 ± 0.13	0.20 ± 0.02

TABLE IV. NA synthesis in and maximum overflow from 12 cat spleens in response to nerve stimulation of different frequencies. Mean values ± SEM. Relation of synthesis to maximal overflow. Multiplying synthesis and overflow values by 9.4 gives an estimate of synthesis and overflow of NA expressed in ng/g w.w./min

No	Stimulation Frequency	Synthesis ng NA/DNA U/min		Overflow ng NA/DNA U/min		Synthesis overflow %	
1	1/sec	0.35		3.74		9.4	
2		0.18		2.46		8.1	
3		0.85		5.16		16.4	
4		0.40		5.41		7.4	
			0.45 ± 0.14		4.19 ± 0.69		10.3 ± 2.1
1	5/sec	0.42		17.20		2.5	
2		0.27		8.61		3.1	
3		0.40		17.90		2.2	
4		0.74		15.10		4.9	
			0.46 ± 0.10		14.68 ± 2.10		3.2 ± 0.6
1	10/sec	0.14		10.47		1.3	
2		0.25		7.69		3.3	
3		0.74		7.79		9.5	
4		0.67		22.80		2.9	
			0.45 ± 0.15		12.19 ± 3.60		4.3 ± 1.8

The total amount of NA recovered in the perfusate from the stimulated portion of the spleen (NA_f) was very large both absolutely and in relation to the calculated starting NA content of the stimulated portion of the spleen (NA_o): 53.0 ± 4.3 % of NA_o (mean ± SEM, n = 12) (*cf.* Table V). The NA content of the stimulated portion of the spleen found at the end of the experiment (NA_e) was always lower than NA_o. However, while there was thus a substantial reduction of the tissue content of NA the depletion was by no means complete (39.0 ± 4.5 % of NA_o, mean ± SEM, n = 12) (*cf.* Table V). Since the depletion was less than NA_f, part of the overflowing NA must have been replaced by newly synthesized neurotransmitter.

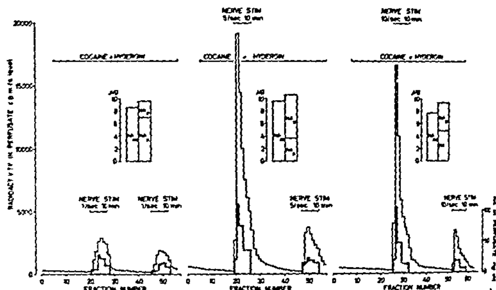


Fig 1 Perfused cat spleens pretreated with malamide and loaded with ³H-dl NA Outflow of radioactivity and of fluorimetrically determined NA (shaded areas) from the spleen resting and in response to nerve stimulation Cocaine + hydergin added to the perfusion medium to inhibit uptake and to facilitate washout of NA released from the sympathetic neurone Calculated starting NA content in stimulated spleen (NA_{c_s}) NA content in stimulated spleen at the end of the experiment NA_s and NA content in perfusate (NA_f)

At 1/sec the peak level of NA overflow was relatively well maintained throughout the stimulation period or decreased only moderately With the higher stimulation frequencies the NA outflow reached a higher peak level, only to fall quite steeply (Fig 1 2)

The peak level of the NA overflow response to a second nerve stimulation period after a resting interval of 20- 30 min was always lower than the peak level in the first stimulation period and closer to the NA level towards the end of the first stimulation period (Fig 1 2

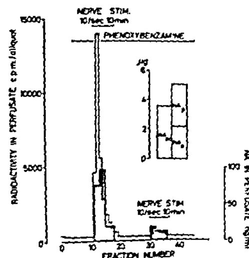


Fig 2 Perfused cat spleen pretreated with malamide and loaded with ³H-dl NA Outflow of radioactivity and of fluorimetrically determined NA (shaded areas) from the spleen resting and in response to nerve stimulation PBA added to the perfusion medium to inhibit uptake and to facilitate washout of NA released from the sympathetic neurone Calculated starting NA content in stimulated spleen (NA_c) NA content in stimulated spleen at the end of the experiment (NA_s) and NA content in perfusate (NA_f)

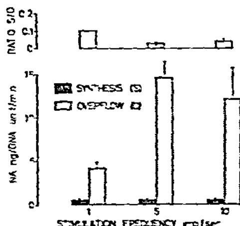


Fig. 3. NA synthesis in perfused cat spleens and maximum NA overflow from the same in response to nerve stimulation of different frequencies. Ratio synthesis maximum overflow. Mean values ± SEM.

neurons of the released NA by addition of PBA or cocaine – Hydergin to the perfusion medium. *De novo* synthesis of NA was estimated as the difference between the total amount of NA found in organ and perfusate and that calculated to be present in the organ at the beginning of the experiment. This calculation in its turn was based on the use of DNA as a reference and on the finding in the present experiments that the NA of the cat spleen was homogeneously distributed between the two main portions of the organ. This observation is somewhat in contrast to that of Dearnaley and Geffen (1966 b) who reported a 10 % difference of the NA content in two portions of cat spleen.

A methodological difficulty in this study was the need to use drugs to block intra axonal enzymatic metabolism of NA (nialamide), to prevent reuptake of liberated, extraaxonal transmitter (PBA, cocaine) and to facilitate its complete and rapid wash-out (PBA, Hydergin). The difficulty consisted in choosing a drug concentration which would produce maximum specific effects without distorting NA synthesis or release because of possible side effects. Such side effects have been reported for PBA, which when administered in a single high dose has been shown to cause release of NA from the perfused cat spleen (Furchgott and Kirpekar 1963, Fischer, Horst and Kopin 1965). Moreover, PBA when added in relatively high concentrations to suspensions of NA storage particles isolated from bovine splenic nerves *in vitro* has been shown both to counteract spontaneous release of NA from the particles (Euler and Lishajko 1962) and to inhibit the last step in NA synthesis from tyrosine (Stjärne and Lishajko 1966). Thus it might be suspected that this drug would distort the normal picture, changing the resting NA leakage from the nerves, possibly decreasing the amount of NA released during nerve stimulation and giving erratically low figures for NA synthesis. However, in the present experiments addition of PBA to the perfusion medium did not change the resting outflow of either radioactive or of fluorimetrically determined NA. Moreover, the drug strongly potentiated the NA overflow response to electrical nerve stimulation (cf. Brown and Gillespie 1957 and others). Finally the figures for NA synthesis obtained in the

present experiments were high when compared to data in the literature (*cf* Spector *et al* 1862 Montanari *et al* 1963 Levitt *et al* 1965 Sedvall *et al* 1968) This suggests that the abovementioned *in vitro* inhibitory effects of PBA on NA synthesis or on the release of NA need not necessarily apply to the intact neuron and might therefore not affect the present preparation This is further supported by the almost identical results obtained in the present series when cocaine + Hydergin were used instead of PBA Cocaine was chosen because it is known to inhibit NA uptake into sympathetic neurons (Whitby Hertting and Axelrod 1960) by blocking the specific uptake mechanism at the level of the axonal membrane (Hillarp and Malmfors 1964) without inhibiting NA biosynthesis (Holtz Oswald and Stock 1960) or affecting the spontaneous release of NA from isolated bovine splenic nerve granules (Euler and Lishajko 1965) Hydergin was given in a small dose about 50 times less than that of PBA just sufficient to block the alpha receptor response in order to facilitate the washout of NA Very much like PBA Hydergin is known to retard the spontaneous NA release from isolated nerve granules (Euler and Lishajko 1966) *although at a concentration much higher than that used in the present experiments* On the other hand Hydergin has been found not to inhibit NA synthesis in bovine splenic nerve homogenate fractions at concentrations which strongly block spontaneous NA release (Stjarne and Lishajko 1966) In agreement with the observations in the PBA experiments addition to the perfusion medium of cocaine + Hydergin did not change the resting NA overflow from the perfused spleen These considerations indicate that PBA cocaine and Hydergin in the concentrations used did not interfere with synthesis or release of NA in the splenic nerves

Another aspect of the drug problem is the question whether the concentrations of PBA or cocaine present in the perfusion medium were sufficient to block completely the NA uptake process at the level of the axonal membrane *In vivo* pretreatment with either PBA or cocaine has been shown to reduce NA uptake in the perfused cat spleen to the same extent as postganglionic denervation (Gillespie and Kirpekar 1965) Moreover in other experiments in this laboratory (Hedqvist unpublished) higher concentrations of PBA and cocaine than those used in the present series were found not to further increase the NA overflow response to nerve stimulation in the isolated perfused cat spleen Thus it appears likely that the inhibition of the NA reuptake into the nerves was nearly complete and that therefore the values for NA in the perfusate in the presence of these drugs accurately reflect the NA release from the nerves

One additional drug problem concerns the use of nialamide to block MAO which was regarded advantageous for the balance study Long term MAO inhibition has been shown to reduce NA biosynthesis possibly by product inhibition of the tyrosine hydroxylation step (Neff and Costa 1966) This might imply that the synthesis values found by us are not representative of the physiological rate of NA synthesis However in our experiments short term MAO inhibition did not alter the normal NA content of the cat spleen (*cf* Malmfors 1964 b) Moreover MAO inhibition is known not to change the NA output from the spleen either resting or in response to

nerve stimulation (Brown and Gillespie 1957) Thus it appears likely that the basic balance between NA synthesis and overflow was not affected by MAO inhibition

Further methodological difficulties were caused by the need to find suitable correction factors for the fluorimetric NA values All of these were corrected for losses on alumina (see under Methods) Moreover the NA values in the perfusate but not in the tissue extracts were in addition corrected for metabolites using results from a chromatographic analysis of the radioactivity appearing in the perfusate The basis for this was the following

Metabolites made up only a small proportion of the radioactivity both in total perfusate and tissue extracts Since MAO was inhibited and since O methylation appears to proceed exclusively extraneuronally (Waltman and Sears 1964 Potter *et al* 1965 Iversen Glowinski and Axelrod 1966) the metabolites in the splenic extracts (less than 4 per cent of the total radioactivity) may be assumed to be derived largely from exogenous NA retained and metabolized extraneuronally Thus correction for these metabolites of the fluorimetrically determined NA figures in tissue extracts did not appear to improve on the accuracy of the estimation of intraneuronal NA On the other hand the metabolites appearing in the perfusate from the spleen may be assumed to be largely derived from NA originally bound in the nerves protected against intraaxonal enzymatic inactivation subsequently released from the nerves as intact NA and metabolized extraneuronally On these grounds it appeared necessary to correct the fluorimetrically determined NA values in the perfusate for metabolites to improve on the accuracy of the estimation of the amounts of NA actually released from the nerves

Methodologically the use of exogenous dl NA administered about 40 min before the beginning of the stimulation period as a tracer to reveal the fate of the endogenous l NA involves a number of difficulties It may be argued that exogenous NA could not equilibrate with the endogenous NA during such a short period of time Moreover the racemic tracer obviously is less suitable to reflect uptake storage release and enzymatic inactivation of the endogenous l NA However l and d NA have been reported to be equally good substrates for catechol O methyl transferase (Axelrod and Tomchick 1958) Furthermore in view of the preferential retention of the l isomer (Maickel Beaven and Brodie 1963 Iversen 1963) there is reason to assume that the majority of the exogenous NA retained in the spleen after 40 min of washing consisted of l NA In fact the close agreement between outflow of radioactive and fluorimetrically determined NA in the present experiments indicates a reasonably complete equilibration between exogenous and endogenous NA Finally since the metabolites represented less than 10 per cent of the total radioactivity in the perfusate any error introduced by this correction factor should be at the most quite small Thus the corrected values for NA in the perfusate should give a reasonably accurate estimate of the total NA given off from the neurons

The values for NA release and synthesis obtained by these methods appear to be in general agreement with findings in other laboratories Thus the NA output per nerve impulse calculated from the peak NA loss from the neurons of the spleen

during the initial part of the stimulation periods compares relatively well with results from the literature (Brown and Gillespie 1957, Kirpekar and Cervoni 1963 Haefely *et al* 1965)

The rapid fading of the neurotransmitter output during continuous nerve stimulation appears to be due to the effective drainage of the 'pool' of NA immediately available to release by nerve impulses in the presence of drugs inhibiting refilling of this "pool" by reuptake of the NA released from the nerves Under these conditions continuous nerve stimulation led to partial depletion of the total NA in the organ However NA amounting to more than one half of the initial level turned out to be resistant to release by nerve stimulation The nature of this functional compartmentalization of the stored NA is presently under study in this laboratory

It must also be pointed out that when reuptake of NA is not inhibited very little NA escapes to the circulation during nerve stimulation at frequencies below 10 per sec (*cf* Brown and Gillespie 1957 and others) Therefore it appears reasonable to assume that the large NA discharge in response to nerve impulses which in our experiments was observed only during the first part of the stimulation periods may represent the normally occurring 'physiological' transmitter release, which in the absence of drugs blocking reuptake is masked because the major proportion of the NA released is retained in the organ, ultimately recaptured into the neurons and reused (Paton 1960 Blakeley *et al* 1964, Stjarne 1964, Brown 1965, Gillespie and Kirpekar 1965, Haefely *et al* 1965 Blakeley and Brown 1966 Hedqvist and Stjärne 1966, Geffen 1967 Folkow *et al* 1968)

The NA synthesis figures obtained in the present series, averaging 250 ng/g tissue/hour, are quite high in comparison to those of Blakeley and Brown (1966) In a study of NA synthesis in the isolated cat spleen perfused with blood without addition of drugs these authors calculated the rate of NA synthesis to 0 3 µg/hour which in a 5 g spleen would correspond to 60 ng/g/ hour In their study intermittent high frequency nerve stimulation was used and the estimation of synthesis was based on determination of steady state output under conditions where the organ content of NA was supposed to remain constant In our experiments the spleen was exposed to prolonged continuous nerve stimulation and drugs were added to the salinic perfusion medium to ensure inhibition of NA reuptake into the neurons as well as its complete and rapid washout The estimation of synthesis was based on a balance calculation after correction for both losses on alumina and for metabolites These may be some of the reasons for the differences observed It may also be pointed out that PBA pretreatment *in vivo* has been observed to produce a marked increase in the NA synthesis in various tissues (Udenfriend 1968)

The high rate of NA synthesis in the present experiments probably represents acceleration from the resting rate level induced by the increased nerve activity (*cf* Andén *et al* 1965 Oliverio and Stjarne 1965 Gordon *et al* 1966 Roth Stjarne and Euler 1966, Sedvall and Kopin 1967) The range of NA synthesis values was almost identical at the different stimulation frequencies This indicates that the acceleration of the NA synthesis induced by increased nerve activity is triggered by

some factor secondary to the depolarization frequency rather than by the depolarization as such (Roth, Stjarne and Euler 1967) Such a factor might be some strategic NA pool which becomes depleted as a result of the increased transmitter discharge

As mentioned above, the purpose of the present investigation was to estimate the physiological discharge of NA in response to nerve impulses, and to find out to what extent local synthesis could replace the NA lost, under conditions where this loss was made permanent by prevention of NA reuptake The results obtained indicate that the average rate of NA synthesis corresponds to only about 6 per cent of the maximum NA discharge from the neurons in response to nerve stimulation With due respect to the technical and theoretical fallacies in this type of calculation discussed above, the striking discrepancy found between the magnitude of trans mitter discharge and the capacity for replacement by local formation of new trans mitter leads to the conclusion that "short term neurotransmitter homeostasis", *i e* the mechanism whereby a constant level of neurotransmitter is maintained in the sympathetic neurons from one moment to the next is not mainly based on *de novo* synthesis, but rather on almost quantitative reuptake and reuse of neurotransmitter (*cf* references above)

However, even under conditions where the recapture mechanism functions optimally, it is obvious that some of the NA released from the axons must be permanently lost, due to removal by the circulation or to local, extraneuronal enzymatic degradation This loss, as well as that due to intraaxonal enzymatic inactivation, requires compensation by *de novo* synthesis During periods of increased nerve activity an increased exposure of the NA released from protective binding in the axon should result in an increased rate of loss of NA from the tissue Adjustment of the rate of synthesis according to the functional state of activity of the organ (Anden *et al* 1965 Oliverio and Stjarne 1965, Roth Stjarne and Euler 1966 Gordon *et al* 1966 Sedvall and Kopin 1967) therefore appears to be the mechanism whereby the long term constancy, (*cf* Kopin *et al*) or in certain organs the controlled rhythmic variation (*cf* Hokfelt 1951, Wurtman and Axelrod 1966 Wurtman, Axelrod and Sedvall 1967, Reis and Wurtman 1968) of the NA store is regulated (long term neuro transmitter homeostasis)

This work was supported by grants from the Swedish Medical Research Council projects No 14X 97 03 No 14X 609 02 and Stiftelsen Lars Hiertas Minne which are hereby gratefully acknowledged

Addendum As emphasized in the discusion the authors are aware of the possibility that the drugs used may interfere with the processes of NA synthesis and release In fact recent ob servations at this laboratory (Hedqvist unpublished) indicate that PBA by a mechanism different from those discussed above may induce an increased NA overflow in response to nerve stimulation

References

ANDEN N E, A CARLSSON N A HILLARP and T MAGNUSSON Noradrenaline release by nerve stimulation of the spinal cord *Life Sci* 1965 *4* 129—132

AXELROD J and R TOMCHICK Enzymatic O methylation of norepinephrine and other catechols *J biol Chem* 1958 *233* 702—705

BLAKELEY, A G H, and G L BROWN Release and turnover of the adrenergic transmitter *In* "Mechanisms of release of biogenic amines Editors U S v Euler S B Uvnas Pergamon Press 1966 185—187

BLAKELEY, A. G. H., G. L. BROWN and L. B. GEFFEN, Uptake and reuse by sympathetic nerves of the transmitter they liberate. *J Physiol (Lond.)* 1964. *173*. 22P—23P.

BROWN, G. L. The release and fate of the transmitter liberated by adrenergic nerves. *Proc roy Soc B* 1965. *162*. 1—19.

BROWN, G. L. and J. S. GILLESPIE. The output of sympathetic transmitter from the spleen of the cat. *J Physiol (Lond.)* 1957. *138*. 81—102.

BURTON, K. A study of the conditions and mechanism of the action of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem J* 1956. *62*. 315—323.

CERVONI, P., S. M. KIRPEKAR and A. SCHWAB. The effect of drugs on uptake and release of catecholamines in the isolated left atrium of the guinea pig. *J Pharmacol exp Ther* 1966. *151*. 195—206.

DEARNALEY, D. P. and L. B. GEFFEN, Depletion of the noradrenaline content of the cat spleen by nerve stimulation. *J Physiol (Lond.)* 1966 a. *182*. 1—42P.

DEARNALEY, D. P. and L. B. GEFFEN. The effect of phenoxybenzamine on depletion by nerve stimulation of the noradrenaline of the cat's spleen. *J Physiol (Lond.)* 1966 b. *184*. 73P—77P.

EULER, U. S. v. and S. HELLNER-BJÖRKMAN, Effect of increased adrenergic nerve activity on the content of noradrenaline and adrenaline in cat organs. *Acta physiol scand* 1955. *33*. Suppl. 118. 17—20.

EULER, U. S. v. and F. LISHAJKO. Improved technique for the fluorimetric estimation of catecholamines. *Acta physiol scand* 1961. *51*. 348—355.

EULER, U. S. v. and F. LISHAJKO. Effect of some drugs on the release of noradrenaline from isolated nerve granules. *Biochem Pharmacol* 1962. *9*. 77—84.

EULER, U. S. v. and F. LISHAJKO. *Effect of drugs on the storage granules of adrenergic nerves. In "Pharmacology of cholinergic and adrenergic transmission"* Proc. 2nd Internat. Pharmacol. Meeting Prague 20—23 August 1963. 245—259. Czechoslovak Medical Press Praha 1965.

EULER, U. S. v. and F. LISHAJKO. Inhibitory action of adrenergic blocking agents on catecholamine release and uptake in isolated nerve granules. *Acta physiol scand* 1966. *68*. 257—262.

FISCHER, J. E., W. D. HORST and I. J. KOPIN. Hydroxylated sympathomimetic amines as false neurotransmitters. *Brit J Pharmacol* 1965. *24*. 477—484.

FOLKOW, B., J. HÄGGENDAL and B. LISANDER. Extent of release and elimination of noradrenaline at peripheral adrenergic nerve terminals. *Acta physiol scand* 1968. *72*. Suppl. 307.

FREDHOLM, B. and G. SEDVALL. Influence of sympathetic nerve stimulation on noradrenaline stores in the rat salivary gland. *Life Sci* 1966. *5*. 2023—2032.

FURCHGOTT, R. F. and S. M. KIRPEKAR. Competition between β-haloalkylamines and norepinephrine for sites in cardiac muscle. *In Proc. First Internat. Pharmacol. Soc. Meeting*. Pergamon Press 1963. *7*. 339—350.

GEFFEN, L. B. Noradrenaline storage, release and inactivation in sympathetic nerves. *Circulat Res* 1967. *21*. Suppl. III. 57—61.

GILLESPIE, J. S. and S. M. KIRPEKAR. The inactivation of infused noradrenaline by the cat spleen. *J Physiol (Lond.)* 1965. *176*. 205—227.

GILLESPIE, J. S. and S. M. KIRPEKAR. The uptake and release of radioactive noradrenaline by the splenic nerves of cats. *J Physiol (Lond.)* 1966. *187*. 51—68.

GORDON, R., S. SPECTOR, A. SJOERDSMA and S. UDENFRIEND. Increased synthesis of norepinephrine and epinephrine in the intact rat during exercise and exposure to cold. *J Pharmacol exp Ther* 1966. *153*. 440—447.

HAEFELY, W., A. HÜRLIMANN and H. THOENEN. Relation between the rate of stimulation and the quantity of noradrenaline liberated from sympathetic nerve endings in the isolated perfused spleen of the cat. *J Physiol (Lond.)* 1965. *181*. 48—58.

HEDQVIST, P. and L. STJÄRNE. The role of the reuptake mechanism in noradrenergic nerves. *Acta physiol scand* 1966. *68*. Suppl. 277. 71.

HILLARP, N.-Å. and T. MALMFORS. Reserpine and cocaine blocking of the uptake and storage mechanism in adrenergic nerves. *Life Sci* 1964. *3*. 703—708.

HÖKFELT, B. Noradrenaline and adrenaline in mammalian tissues. *Acta physiol scand* 1951. *25*. Suppl. 92.

HOLTZ, P., W. OSSWALD and K. STOCK. Über die Beeinflussung der Wirkungen sympathomimetischer Amine durch Cocain und Reserpin. Naunyn-Schmiedebergs *Arch exp Path Pharmak* 1960. *239*. 14—28.

IVERSEN, L. L. The uptake of noradrenaline by the isolated perfused rat heart. *Brit J Pharmacol* 1963. *21*. 523—537.

IVERSEN, L. L., J. GLOWINSKI and J. AXELROD. The physiological disposition and metabolism of norepinephrine in immunosympathectomized animals. *J Pharmacol exp Ther* 1966. *151*. 273—284.

KERNELL D and G SEDVALL Reduction of the noradrenaline content of skeletal muscle by sympathetic stimulation *Acta physiol scand* 1964 *61* 201—202

KIRPEKAR S M and P CERVONI Effect of cocaine phenoxybenzamine and phentolamine on the catecholamine output from spleen and adrenal medulla *J Pharmacol exp Ther* 1963 *142* 59—70

KOPIN I J and E GORDON, Origin of norepinephrine in the heart *Nature* (Lond) 1963 *199* 1289

KOPIN I J, E K GORDON and D HORST Studies of uptake of l norepinephrine ^{14}C *Biochem Pharmacol* 1965 *14* 753—759

KOPIN I J G R BREESE K R KRAUSS and V K WEISE Selective release of newly synthesized norepinephrine from the cat spleen during nerve stimulation *J Pharmacol exp Ther* 1968 *161* 271—278

LEVITT M S SPECTOR A SJOERDSMA and S UDENFRIEND Elucidation of the rate limiting step in norepinephrine biosynthesis in the perfused guinea pig heart *J Pharmacol exp Ther* 1965 *148* 1—8

LUCO J V and F GONI Synaptic fatigue and chemical mediators of postganglionic fibres *J Neurophysiol* 1948 *11* 497—500

MAICKEL, R P M A BEAVEN and B B BRODIE Implications of uptake and storage of norepinephrine by sympathetic nerve endings *Life Sci* 1963 *2* 953—958

MALMFORS T Release and depletion of the transmitter in adrenergic terminals produced by nerve impulses after the inhibition of noradrenaline synthesis or reabsorption *Life Sci* 1964a *3* 1397—1402

MALMFORS T Studies on adrenergic nerves *Acta physiol scand* 1964b *64* Suppl 248

MONTANARI, R. E COSTA M A BEAVEN and B B BRODIE Turnover rates of norepinephrine in hearts of intact mice rats and guinea pigs using tritiated norepinephrine *Life Sci* 1963 *2* 232—240

NEFF N H and E. COSTA, The influence of monoamine oxidase inhibition on catecholamine synthesis *Life Sci* 1966 *5* 951—959

OLIVERIO A and L. STJARNE Acceleration of noradrenaline turnover in the mouse heart by cold exposure *Life Sci* 1965 *4* 2339—2343

PATON W D M *In* "Adrenergic Mechanisms Ciba Found Symp Ed G E W Wolstenholme and M O Conner London Churchill 1960 124—127

POTTER L. T T COOPER V L. WILLMAN and D E. WOLFE Synthesis binding release and metabolism of norepinephrine in normal and transplanted dog hearts *Circulat Res* 1965 *16* 468—481

REIS D J and R J WURTMAN Diurnal changes in brain noradrenaline *Life Sci* 1968 *7* 91—98

ROTH R H L STJARNE and U S v EULER Acceleration of noradrenaline biosynthesis by nerve stimulation *Life Sci* 1966 *5* 1071—1075

ROTH R H L. STJARNE and U S v EULER, Factors influencing the rate of norepinephrine biosynthesis in nerve tissues. *J Pharmacol exp Ther* 1967 *158* 373—377

SEDVALL G and I J KOPIN Acceleration of norepinephrine synthesis in the rat submaxillary gland *in vivo* during sympathetic nerve stimulation *Life Sci* 1967 *6* 45—51

SEDVALL G V K WEISE and I J KOPIN The rate of norepinephrine synthesis measured *in vivo* during short intervals Influence of adrenergic nerve impulse activity *J Pharmacol exp Ther* 1968 *159* 274—282

SPECTOR S K MELMON and A SJOERDSMA Evidence for rapid turnover of norepinephrine in rat heart and brain *Proc Soc exp Biol* N Y 1962 *111* 79—81

STJARNE L. Studies of catecholamine uptake storage and release mechanisms *Acta physiol scand* 1964 *62* Suppl 228

STJARNE L and F LISHAJKO Drug induced inhibition of noradrenaline synthesis *in vitro* in bovine splenic nerve tissue *Brit J Pharmacol* 1966 *27* 398—404

STJARNE L and F LISHAJKO Localization of different steps in noradrenaline synthesis to different fractions of a bovine splenic nerve homogenate *Biochem Pharmacol* 1967 *16* 1719—1728

THOENEN H A. HURLIMANN and W HAEFELY The effect of postganglionic sympathetic stimulation on the isolated perfused spleen of the cat *Helv physiol pharmacol Acta* 1963 *21* 17—26

UDENFRIEND S Physiological regulation of noradrenaline biosynthesis *In* Ciba Foundation Study Group on Adrenergic Neurotransmission London 1968

WALTMAN S and M SEARS Catechol-O methyl transferase and monoamine oxidase activity in the ocular tissues of albino rats *Invest Opthal* 1964 *3* 601—605

WHITBY L G G HERTTING and J AXELROD Effect of cocaine on the disposition of noradrenaline labelled with tritium *Nature* (Lond) 1960 *187* 604—605

WURTMAN R J and J AXELROD A 24 hour rhythm in the content of norepinephrine in the pineal and salivary glands of the rat *Life Sci* 1966 *5* 665—669

WURTMAN R J J AXELROD and G SEDVALL, Photic and neural control of the 24 hour norepinephrine rhythm in the rat pineal gland. *J Pharmacol exp Ther* 1967 *157* 487—492

Acta physiol. scand. 1969. 76 284—287

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Pulmonary Diffusing Capacity Limiting Human Performance at Altitude

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Received 3 September 1968

Abstract

Blomqvist, G, R L Johnson Jr and B Saltin *Pulmonary diffusing capacity limiting human performance at altitude* Acta physiol scand 1969 76 284—287

In 6 internationally successful endurance athletes measurements of oxygen uptake and pulmonary diffusion capacity at maximal bicycle exercise were performed at sea level and after 3 weeks stay at 2250 m altitude (Mexico City) Maximal oxygen uptake was 10—17 % lower at altitude and this reduction was related to the ratio at sea level between D_{LCO}/max $\dot{V}_{O_2}$ The findings demonstrated that there is a physiological basis not only for a more marked reduction of max $\dot{V}_{O_2}$ in welltrained groups at altitude but also for the observed large differences in performance at altitude between athletes of equal capability at sea level

Diffusion capacity of the lung does not limit maximal oxygen uptake in normal subjects at sea level (Johnson 1967) However, based on the same evidence measurements of membrane diffusing capacity pulmonary capillary blood volume and cardiac output at sea level indicate that diffusing capacity should become an increasingly important factor at altitudes above 1500 m, particularly at high pulmonary capillary blood flows Previous studies suggest that a decrease of the oxygen content of ambient air may be associated with a larger reduction of maximal oxygen uptake in well trained groups than in groups with average cardiovascular capacity (Saltin 1967) but conflicting evidence has also been presented (Faulkner *et al* 1968) The present study of a group of internationally successful endurance athletes was undertaken to investigate quantitatively the relation between diffusing capacity of the lung and changes in performance between sea level and altitude

Subjects and methods

Included in the study were 6 members of the Swedish national bicycle team taking part in the [illegible] determined [illegible]

TABLE I Individual data

Subject	Age yrs Height cm Weight kg	Place of study[1]	Measurements during maximal work $\dot{V}_{O_2}$ STPD l/min	Heart rate beats/min	Q l/min	D_{LCO} ml/min / mmHg	$\dot{V}_E$ BTPS l/min	Hgb g %	Hct %
A	23	S	5.14	184			187.2	14.3	43
	183	MC	4.15	179			194.0	14.9	44
	65	D	4.94	179	28.7	57.7	194.3	15.1	44
B	27	S	6.18	187			205.4	14.8	44
	189	MC	4.94	187			236.8	16.2	46.5
	75	D	6.10	185	36.5	69.5	219.8	15.2	44
C	25	S	5.56	190	31.6[2]		178.4	14.3	43
	188	MC	4.80	186			218.9	15.3	44
	75	D	5.42	189	27.1	66.8	201.3	15.3	44
D	26	S	5.60	183	30.9[2]		170.9	14.8	45
	179	MC	4.57	183			196.2	15.9	48
	72	D	5.31	178		65.2	194.3	16.0	47
E	25	S	5.84	192			169.5	14.3	42
	187	MC	4.87	182			186.3	15.9	47
	74	D	5.43	183	32.1	71.2	171.4	15.7	46
F	24	S	5.27	171	29.6[2]		153.9	15.1	44
	180	MC	4.40	170			193.1	16.0	47
	77.5	D	5.07	171	26.9	65.0	177.2	15.7	46.5

[1] S = Stockholm MC = Mexico City D = Dallas

[2] The dye dilution technique was used for this cardiac output determination

modified for simultaneous cardiac output (Q) measurements by the acetylene technique (Saltin *et al* 1958) Rebreathing was performed from the functional residual capacity with an initial volume in the *rebreathing bag approximately*
rate was determined from ECG re[illegible]
meter Measurements were made [illegible]
subjects could tolerate for 6 min [illegible]
(Dallas) a 3 week stay in Mexico [illegible]
of the 3 week period Diffusion capacity was measured on the first day after return to sea level (Dallas)

Results and Discussion

The results presented in Table I and Fig 1 indicate that diffusion capacity of the lung is an important limiting factor at a barometric pressure of 580 mm Hg It is apparent that the magnitude of the decrease in maximal $\dot{V}_{O_2}$ at altitude is determined by the relation between diffusing capacity and the potential rate of oxygen uptake, i e the ratio D_{LCO} maximal $\dot{V}_{O_2}$ at sea level

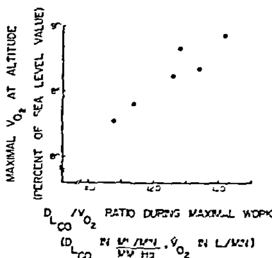


Fig. 1. The relationship between reduction in maximal $\dot{V}_{O_2}$ at 2,250 m.a.s.l. and the ratio between the pulmonary diffusing capacity for CO and oxygen uptake at maximal exercise in 6 athletes.

Maximal $\dot{V}_{O_2}$ at sea level was in all 6 subjects slightly lower after than before the stay at altitude. The difference was probably related to a reduced intensity of training at altitude. Either set of sea level data might have been used to demonstrate the relationship shown in Fig. 1. A similar correlation is also present between maximal $\dot{V}_{O_2}$ at altitude and the ratio $D_{L_{CO}}$: maximal cardiac output at sea level.

In young adults a conditioning program induce a larger increase in max $\dot{V}_{O_2}$ than in $D_{L_{CO}}$ (Saltin et al. 1968). This is in accordance with the findings that individuals with a large aerobic work power during maximal exercise have a smaller margin to the upper limit of the lungs to transfer oxygen (Shepard *et al.* 1967). The ratio for $D_{L_{CO}}$: max $\dot{V}_{O_2}$ had a mean value of 12.2 in the present study of endurance athletes compared with 13 in trained students (Holmgren and Åstrand 1966), 14 in untrained students (Saltin *et al.* 1968) and 15 in untrained female students (Holmgren and Åstrand 1966). These findings may then explain the observations that the largest reduction in max $\dot{V}_{O_2}$ at altitude is found in the most well trained groups (Saltin 1967, Grover *et al.* 1967, Faulkner *et al.* 1968). It is worth noticing, however, that is the ratio between $D_{L_{CO}}$ and max $\dot{V}_{O_2}$ that is the crucial factor for the reduction in max $\dot{V}_{O_2}$ at altitude and not an extremely high max $\dot{V}_{O_2}$ per se. Thus the present findings may also give the physiological explanation for the observed large differences in performance at altitude between athletes of equal capacity at sea level.

This study was supported by grants from the Swedish Sports Federation, Pol. [illegible] and USPHS HE 0[illegible]. Technical assistance was given by Mrs Inger Hallbäck and Mr Abraham [illegible].

References

Faulkner, J. A., J. Kollias, C. B. Favour, E. R. Buskirk and B. Balke. Maximum aerobic capacity and running performance at altitude. *J. appl. Physiol.* 19[illegible] 24 [illegible].
Holmgren, A. and P-O Åstrand. D_L and the dimensions and functional capac[illegible] [illegible] in [illegible]. *J. appl. Physiol.* 19[illegible] 21 1463–14[illegible].

JOHNSON R L. Jr, Pulmonary diffusion as a limiting factor in exercise stress *Circulat Res* 1967 *20* I 154 I 160

LEWIS B M, T H LIN, F E NOE and E J HAYFORD-WELSING The measurement of pulmonary diffusing capacity for carbon monoxide by a rebreathing method *J clin Invest* 1959 *38* 2073—2086

SALTIN, B, Aerobic and anaerobic work capacity at 2 300 meters *Med Thorac* 1967 *24* 205—210

SALTIN, B, R F GROVER C G BLOMQVIST, L H HARTLEY and R L. JOHNSON JR., Maximal oxygen uptake and cardiac output after two weeks at 4 300 meters *J appl Physiol* 1968 *25* 400—409

SALTIN B G BLOMQVIST J H MITCHELL, R JOHNSON JR K WILDENTHAL and C B CHAPMAN Response to submaximal and maximal exercise after bedrest and training *Circulation* 1968 *38* Suppl VII

SHEPARD R. H E J M CAMPELL H B MARTIN and T ENNS, Factors affecting the pulmonary dead space as indicated by single breath analysis *J appl Physiol* 1957 *11* 241—245

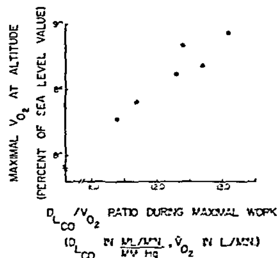


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References

FAULKNER, J A., J [illegible] C B FAVOUR E. R BUSKIRK and B BALKE. Maximum aerobic capacity and running performance at altitude *J appl Physiol* 1968. 24 685—691
HOLMGREN A. and P O ÅSTRAND D_L and the dimensions and functional capacities of the O_2 transport system in humans *J appl Physiol* 1966 21 1463—1470

JOHNSON, R. L. Jr, Pulmonary diffusion as a limiting factor in exercise stress *Circulat Res* 1967 *20* I 154-I 160
LEWIS, B M, T H LIN, F E NOE and E J HAYFORD-WELSING The measurement of pulmonary diffusing capacity for carbon monoxide by a rebreathing method *J clin Invest* 1959 *38* 2073—2086
SALTIN, B, Aerobic and anaerobic work capacity at 2 300 meters. *Med Thorac* 1967 *24* 205—210
SALTIN, B, R F GROVER, C. G BLOMQVIST, L H HARTLEY and R L. JOHNSON JR, Maximal oxygen uptake and cardiac output after two weeks at 4 300 meters *J appl Physiol* 1968 *25* 400—409
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Acta physiol. scand. 1969. 76. 288—298

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Studies on the Histamine Releasing Effect of Bee Venom Fractions and Compound 48/80 on Skin and Lung Tissue of the Rat

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Received 4 September 1968

Abstract

Fredholm, B and Ö Haegermark *Studies on the histamine releasing effect of bee venom fractions and compound 48/80 on skin and lung tissue of the rat* Acta physiol scand 1969 *76* 288—298

Two bee venom fractions, F [illegible] were shown to release histamine fr[illegible]se A containing fraction, and F I[illegible] the time course of the histamine [illegible] The results with F II and compound 48/80 were similar, suggesting that the two substances activate the same kind of release mechanism It is concluded that F II is not related to the earlier described bee venom polypeptides melittin and apamin The mode of action of phosphatidase A was distinctly different from that of F II or compound 48/80 The results are consistent with the assumption that it acts in a "non-specific" way by hydrolysing tissue phosphatides to lyso compounds, which in turn damage the tissue

Bee venom has been shown to contain several factors which can release histamine in various animal species Such agents are phosphatidase A melittin (Riley 1958 Rothschild *1965*), and a recently-described venom fraction, *F II* (*Fredholm 1966* Fredholm and Haegermark 1967) It has been suggested that phosphatidase A causes histamine release by hydrolysing tissue phosphatides to fatty acids and lyso compounds, which, in turn attack histamine-storing cells (Feldberg, Holden and Kellaway 1938, Trethewie *1939* Rothschild *1965*) Melittin has high surface activity (Habermann 1958), and its effect on mast cells is probably due to this property (Riley 1958, Habermann 1965 Rothschild 1965) The effect of F II on rat peritoneal mast cells has several characteristics in common with that of the histamine liberator compound 48/80 (Fredholm and Haegermark 1967) and thus differs from both phosphatidase A and melittin

The aim of the present work was to study the histamine releasing effects of the two bee venom fractions F I (phosphatidase A) and F II on skin and lung tissue of the rat and to compare the effects with those of compound 48/80

Methods

Fractionation of bee venom

[illegible]

Preparation of skin and lung tissue

Sprague Dawley rats on both sexes (350—500 g) were used They were anaesthetized with [illegible] cised, and freed from subcutaneous fat The skin was cut with scissors into 3×3 mm pieces (weighing about 20 mg) This size was chosen because preliminary experiments had shown that cutting into smaller pieces increased the spontaneous release considerably The cut tissue was thoroughly rinsed with salt solution and allowed to stand at 4° C for 30 min prior to a final wash at 37° C for 2 min It was blotted dry with filter paper and divided into 0 25 g samples The samples were placed in 50-ml Erlenmeyer flasks and suspended in 2 25 ml of salt solution

Lung tissue Four animals were used in each experiment The lungs and heart were excised together To remove blood from the lungs they were perfused via the pulmonary artery with 20 ml of buffered salt solution The heart was removed, and the lung lobes dissected free from visible bronchi The tissue was cut into pieces weighing 5—10 mg, and then treated as described above for skin

Incubation technique

[illegible] of 0 1 N HCl and heated in [illegible] r centrifugation at 1000×g [illegible] the histamine remaining in [illegible] individual experiments, the release was expressed as a percentage of the total amount of histamine in the sample

Histamine assay

Histamine was determined fluorometrically according to Shore Burkhalter and Cohn (1959), using an Aminco-Bowman spectrophoto-fluorometer and 10 mm cuvettes

Electrophoresis

Low voltage paper [illegible] wide strips of Wh[illegible] buffer The curren[illegible] papers were cut transversely into [illegible] pieces which were eluted with 0 01 M NH_4HCO_3

Determination of partition ratios of compound 48/80

The partition ratio of compound 48/80 in a two-phase system was determined using a method similar to that described by Brodie and Udenfriend (1945) The releaser was dissolved in 0 01 M NH_4HCO_3, pH 7 4 to give a concentration of 1 mg/ml. To a 5 ml sample of this solution an equal volume of the organic solvent (heptane or chloroform) was added and the tube was shaken for 2 min at room temperature After centrifugation, the separated phases were evaporated to dryness (water phase freeze dried and organic phase evaporated under nitrogen at room temperature) The residues were weighed and the histamine releasing effect of the recovered material [illegible] neal mast cells as described by Fredholm and Haegermark (1967)

Materials

A bee venom preparation (hereafter referred to as bee venom) obtained by precipitation with picric acid and chromatography on Amberlite IRC-50 and *compound 48/80* were generously supplied by Dr B Hogberg AB Leo Halsingborg Sweden *Lecithin* (for the phosphatidase A assay) *was prepared and kindly supplied by Dr A Wretlind National* Institute of Public Health Stockholm Sweden

Results

Histamine content and spontaneous release

The histamine content of the rat skin ranged from 10 to 30 μg/g (n=32) and of the lung from 5 to 12 μg/g tissue (n=25) (wet weight after cutting and rinsing the tissue)

The spontaneous release of histamine during the incubations varied considerably *between individual experiments During 30 min incubation at 37° C without* liberator the mean release from skin was 17 9 (range 11 0—28 8 n=59) per cent and from lung tissue 10 5 (range 3 8—15 6 n=44) per cent

Dose response relationships

The tissue was incubated at 37° C for 30 min with bee venom F I, F II or compound 48/80 in concentrations ranging from 0 1 to 100 μg/ml For skin the dose release curves were very flat for all agents tested The percentage histamine released was of the same order of magnitude for the two types of tissue, but due to high spontaneous leakage from the skin the net release from that tissue was lower than from lung *e g* with bee venom 100 μg/ml 25 per cent as compared to 35 per cent in the lung with 48/80 100 μg/ml 11 per cent in skin and 29 per cent in lung tissue

Both bee venom fractions caused histamine release and the release curves ran almost parallel F I being the more potent of the two Neither of the fractions showed as high activity as unfractionated bee venom (Fig 1 and 2)

Solubility characteristics of compound 48/80

The poor release from skin might be explained by bad penetration into the tissue fragments due to low fat solubility of the releaser To investigate this possibility the distribution ratio of compound 48/80 was determined in a two-phase system consisting of an aqueous solution of 0 01 M NH_4HCO_3 pH 7 4 and an organic solvent (heptane or chloroform) In the water/chloroform system the releaser was about evenly distributed between the two phases while in water heptane it was almost exclusively found in the aqueous phase (Table I)

Time course of the histamine release

Both compound 48/80 and F II have been shown to cause a rapid release of histamine from rat peritoneal mast cells (Moran Uvnas and Westerholm 1962, Fredholm and Haegermark 1967) To our knowledge there are no data available on the time course of the release induced by any of these agents from other rat tissues

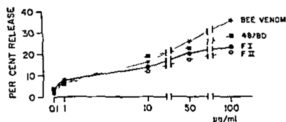


Fig 1 Concentration response curves for histamine release from lung tissue Each point represents the mean value of 3—6 expts S E <15 % of the mean

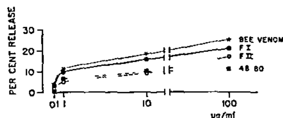


Fig 2 Concentration response curves for histamine release from skin Each point represents the mean value of 3—7 expts S E <15 % of the mean

In the present work the histamine release was followed during the first 60 (in some experiments 180) min of exposure of the tissues to the various releasers at 37° C The spontaneous release was followed in parallel samples

Lung tissue (Fig 3) In the first series of experiments the release was followed for 60 min after the addition of the releaser It was found that the rate of release was initially faster with F II than with F I with F II the maximal release value was reached after only 5 min incubation while F I had liberated only about 50 per cent of the 1 hour value at this time In the samples incubated with F I the release continued although the rate decreased after 10 min and stabilized at a new level which was constant during the rest of the observation period Compound 48/80 liberated histamine at a rate similar to that of F II In order to further illustrate the difference between F I and F II experiments were also performed in which the release was followed for 180 min The results confirmed those of the 1 hour experiments with

TABLE I Distribution of compound 48 80 between lipid solvents and water at pH 7 4 The results are expressed as the ratio of the amount of compound in the organic phase to the total amount

Exp no	System	Recovery per cent	Partition ratio
1	chloroform/water	99	0 46
2		106	0 52
3		108	0 40
4	heptane water	100	0 025
5		101	0 012

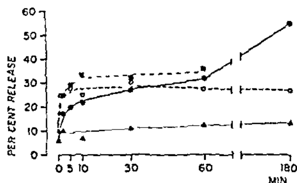


Fig 3 Time course of histamine release from lung tissue, induced by F I, ●——●, F II ○- - -○ and compound 48/80, ■— — —■ Concentration of releaser 50 µg/ml Spontaneous release, ▲ ▲ Values from 2 (48/80) or 4 (F I F II) expts are included in each curve

F I there was a continuous and linear (the linearity is not apparent from the figure as the diagram is broken) release during the period 10—180 min, whereas with F II no further release occurred after the initial 5—10 min

Skin (Fig 4) In contrast to the findings with lung tissue, the spontaneous release from skin increased considerably with increasing incubation time, a fact which may have obscured differences between the release curves for the various agents After 60 min it had reached 25 per cent of the total histamine content, and it was considered useless to extend the observation time any further

With all three liberators the main net release from skin occurred within the first 10 min of incubation and there were no obvious differences between the curves

Influence of metabolic inhibitors and preheating of the tissue

Enzyme inhibitors, and heat treatment of the tissue are known to block the effect of certain histamine releasers, such as antigen or compound 48/80 (Mongar and Schild 1955 1957 Mota and Ishii 1960) The effect of F II on rat peritoneal mast cells was also found to be impaired by metabolic inhibitors (Fredholm and Haegermark 1967), while the action of the bee venom fraction melittin and that of phosphatidase A was not affected by the inhibitor dinitrophenol (Rothschild 1965) In the present work the effect of potassium cyanide and dinitrophenol was studied The tissue was incubated with the inhibitor for 10 min and, after addition of releaser, the incubation was continued for another 20 min It was found that the release induced both by compound 48/80 and by F II was strongly inhibited by

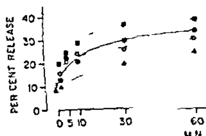
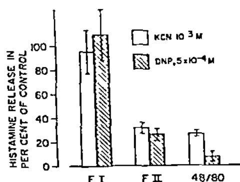


Fig 4 Time course of histamine release from skin induced by F I ●——● F II ○- - -○, and compound 48/80, ■— — — —■ Concentration of releaser 50 µg/ml Spontaneous release ▲ ▲ Each curve represents the mean of 2 expts.



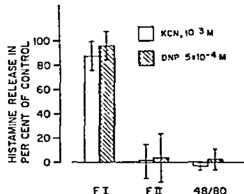


Fig 5 Fig 6

Fig 5 Influence of metabolic inhibitors on the histamine release from lung tissue, induced by F I, F II, and compound 48 80 Concentration of releaser 10 µg/ml The spontaneous release was deducted before calculation Each column represents the mean of 3—4 expts, vertical bars indicate standard errors

Fig 6 Influence of metabolic inhibitors on the histamine release from skin, induced by F I, F II, and compound 48/80 Concentration of releaser 10 µg/ml The spontaneous release was deducted before calculation Each column represents the mean of 4 expts, vertical bars indicate standard errors

either inhibitor, while no significant effect was observed on the histamine release induced by F I (Fig 5 and 6)

"Spontaneous" release was determined both in the presence and in the absence of the inhibitors In several experiments this release was lower in inhibitor-treated samples than in non treated ones, but the difference was not statistically significant

Heating of the skin or lung tissue at 45° C for 15 min before exposure to F II or compound 48/80 reduced the release induced by either agent (Table II)

Paper electrophoresis of F II

F II was subjected to low voltage paper electrophoresis at pH values ranging from 7 0 to 12 25 The histamine releasing material was strongly basic, only at pH 12 25 did it remain at the starting point Some spreading of the activity which occurred when non treated paper was used was probably due to adsorption, as it could be reduced by pretreatment of the paper with 0 5 per cent Tween 20 (*cf* Zeva and Spitznagel 1966)

In vivo effect of F II in the mouse

Bee venom is known to possess excitatory effects on the central nervous system in several species including dog and mouse (Langer 1897, Hahn and Ostermayer 1936) This effect has recently been attributed to a basic polypeptide fraction called apamin (Habermann and Reiz 1964, 1965) The isoelectric point of this fraction is around 12, *i e* about the same as that of F II To see whether F II had any apamin-like activity it was injected intravenously in two series of mice (NMRI strain) In

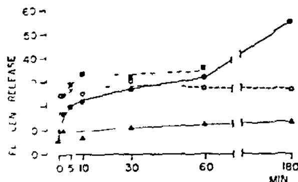


Fig 3 Time course of h[illegible] release from lung tissue, ind[illegible] F I, ●——● F II ○– and compound 48/80 ■– [illegible] Concentration of releaser 50 [illegible] Spontaneous release ▲ Values from 2 (48/80) or [illegible] F II) expts are included [illegible] curve

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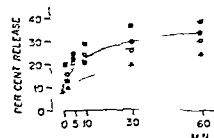


Fig 4 Time course of histamine rel[illegible] induced by F I ●——● F II ○ [illegible] compound 48/80, ■–·–·–■ [illegible] releaser 50 µg/ml Spontaneous rele[illegible] ▲ Each curve represents the mean of 2 ex[illegible]

more susceptible to the action of the liberators The poor release obtained even with the highest concentration of compound 48/80 is in agreement with earlier reports on the release both from minced skin (Rothschild 1965) and minced lung tissue (Diamant and Uvnas 1961) by this agent. Seemingly contradictory to the results with skin are the earlier observations that in perfusion experiments or *in vivo*, histamine is readily released from rat skin by small amounts of compound 48/80 (Feldberg and Mongar 1954, Perry 1956) One explanation for this discrepancy might be that the reactivity of the mast cells was impaired during the preparation of the tissue which usually required 1 1/2—2 hours This possibility is supported by the observation that aging of rat skin caused a reduction of subsequent histamine release induced by compound 48/80 (Yamasaki and Endo 1967) An alternative explanation is that in incubation experiments compound 48/80 can not diffuse into the tissue to reach the target cell As one approach to the question of diffusion of the releaser we determined the distribution ratio for compound 48/80 between water and an organic phase Those experiments revealed that in a water/heptane system practically no releaser entered the organic phase, *i e* compound 48/80 is almost insoluble in a non polar solvent It has been shown that in *intact skin* the rate of penetration of various substances is proportional to their lipid solubility (Treherne 1956) Although the experimental conditions differed from those in intact skin the results may indicate that the solubility characteristics of compound 48/80 *disfavour its penetration into the tissue fragments so that it only reaches mast* cells located close to the surface. The situation is apparently different in the lung where the release was compatible with that obtained in perfusion experiments (Feldberg and Mongar 1954) This may be due to the loose structure of that tissue which allows a better penetration of the releaser

The rate of histamine release was best studied in lung tissue as in skin a continuous spontaneous leakage of histamine during the incubation prevented a valid interpretation of the release The histamine release induced both by F II and compound 48/80 from lung tissue was completed within 5—10 min. Considering the time required for diffusion in a preparation like this it is conceivable that the actual release process in the mast cell has the same rapid course as was reported for the effect of F II and compound 48/80 on suspended rat peritoneal mast cells, in that preparation the release was finished within 20 seconds (Fredholm and Haegermark 1967 It may be recalled in this context that when histamine release is induced from a similar lung tissue preparation by a polypeptide found in *Ascaris suis*, the release curve has approximately the same appearance as in the present work (Diamant 1961)

It is well known that the effect of compound 48/80 on rat mast cells or tissues is influenced by physical or chemical agents which interfere with metabolic processes in the cells for references see Uvnas 1961) The present finding that the release induced by both F II and compound 48/80 was inhibited by potassium cyanide, dinitrophenol or preheating the tissue at 45° C lends further support to the idea that the release mechanisms of the two agents are of the same kind The effect of

TABLE II Histamine release induced by F II or compound 48/80 from tissue heated at 45° C before the incubation The release is expressed as a percentage of the release from non heated tissue Spontaneous release was deducted before calculation Concentration of releaser 10 μg/ml

Exp no	Tissue	Release from preheated tissue in per cent of release in controls	
		F II	48/80
1	lung	67	44
2	,,	38	—
3	,,	47	0
4	,,	30	0
5	skin	8	0
6	,,	61	0
7	,,	11	—
8	,,	3	0

all nine animals received 4 mg/kg and six 40 mg/kg b w Controls were given 0 9 per cent NaCl The injection volume was 0 1 ml The animals were observed for three days after the injections Those who had received 4 mg/kg behaved perfectly normally throughout the observation period. In the animals that had received 40 mg/kg there was a symptom free period of about 15 min followed by the development of marked cyanosis ear erythema oedema of the feet, breathing difficulty and general weakness Four of them died within one hour after the injection. One showed moderate signs of hyperexcitability on the first day after the injection and partial hind leg paralysis which persisted during the whole observation period One of the animals recovered completely within one day

Discussion

The bee venom fraction F II a basic polypeptide with a molecular weight between 1000 and 5000 Fredholm 1966) has previously been shown to cause histamine release from rat peritoneal mast cells in suspension, while the fraction F I (phosphatidase A has only a very weak effect on such cells It was suggested that F II acts by triggering an energy-dependent enzymatic release mechanism in the mast cell in a way similar to the histamine liberator compound 48/80 or antigen Fredholm and Haegermark 1967 In the present study it was found that both F I and F II caused histamine release from skin and lung tissue of the rat and the results give support to the idea that F II and compound 48/80 activate the same kind of release mechanism

The dose-response curves revealed that none of the agents released more than a small fraction of the histamine from skin and that the lung tissue was somewhat

capillata (Uvnäs 1960) For the *Ascaris* and *Cyanea* polypeptides it has been shown that their action involves energy-dependent mechanisms and it may be that in respect of histamine release F II belongs to this group of compounds. Phosphatidase A acts apparently in another way as its effect is not influenced by agents interfering with metabolic processes. This observation is consistent with the assumption that the histamine release caused by phosphatidase A is due to the effect of lyso compounds resulting from enzymatic breakdown of tissue phosphatides reactions that do not need metabolic energy

Note added in proof Recently Breithaupt and Habermann (1968) presented important new information about the polypeptide contained in F II

This investigation was supported by grants from Karolinska Institutet, from Riksförbundet mot Allergi, from Svenska Läkaresällskapet and from the National Institutes of Health, U.S.A. (Grant No. AM 04063-08)

The skilful technical assistance of Mrs Birgitta Ask and Miss Ann-Christine Jarnros is gratefully acknowledged.

References

ARCHER, G T., The release of histamine from the mast cells of the rat. *Aust J exp Biol. med Sci* 1959 *37* 383—390

BREITHAUPT H. and E. HABERMANN Mastzelldegranulierendes Peptid (MCD-Peptid) aus Bienengift Isolierung biochemische und pharmakologische Eigenschaften. *Naunyn-Schmiedebergs Arch Pharmak exp Path* 1968. *261* 2[illegible]—2[illegible]0

BRODIE B B and S UDENFRIEND The estimation of basic organic compounds and a technique for the appraisal of specificity *J biol Chem* 1945 *158* 705—714

DIAMANT B Histamine release elicited by extracts from *Ascaris suis*—influence of oxygen lack and glucose *Acta physiol. scand* 1961 *57* 8—22.

DIAMANT B. and B. UVNÄS Evidence for energy requiring processes in histamine release and mast cell degranulation in rat tissues induced by compound 48/80 *Acta physiol. scand* 1961 *53* 315—329

FELDBERG W H F HOLDEN and C. H. KELLAWAY The formation of lysocithin and of a muscle-stimulating substance by snake venoms. *J Physiol.* (Lond.) 1938 *94* 237—243

FELDBERG W and J L. MONGAR, Comparison of histamine release by compound 48/80 and octylamine in perfused tissues *Brit J Pharmacol.* 1954 *9* 197—201

FINKE S R. G B PHILLIPS and E. MIDDLETON JR. Histamine release from human leukocytes by lysolecithin and the effect of *in vitro* anaphylaxis on leukocyte lipids. *J Lab clin Med* 1966 *67* 601—609

[illegible]

scand 1969 *76* 446—457

[illegible] E. Zur Wirkung tierischer Gifte und von Lysocithin auf Grenzflächen. *Z. ges exp Med* 1958 *130* 19—23

HABERMANN E. Recent studies on *Hymenoptera* venoms In *Recent Advances in the Pharmacology of Toxins* Ed Raudonat Pergamon Press. 1965 pp 53—62

HABERMANN E. and K.-G REIZ Apamin ein basisches zentral erregendes Polypeptid aus Bienengift *Naturwissenschaften* 1964 *51* 61

HABERMANN E. and K.-G REIZ Ein neues Verfahren zur Gewinnung der Komponenten von Bienengift, insbesondere des zentralwirksamen Peptids Apamin. *Biochem Z* 1965 *341* 451—466

HAHN G and H LEDITSCHKE Ueber das Bienengift IV Mitteilung Gewinnung beider Giftkomponente durch Dialyse *Ber dtsch chem Ges* 193[illegible] *70* 163[illegible]—1644

HAHN G and H. OSTERMAYER Ueber das Bienengift I Mitteilung *Ber dtsch chem. Ges.* 1936 *69* 2407—2419

F II i th s c fferent from that of the bee venom fraction melittin (Neumann and Habermann 1954), whose action is not inhibited by dinitrophenol and which probably acts in a "non-specific" way by virtue of its high surface activity (Riley 1959, Habermann 1965, Rothschild 1965)

The histamine release by F I was of another character than that induced by F II c pound 48/80 It was not blocked by metabolic inhibitors or heat treatment the release had a slow course and continued during the whole incubation period 3 t n These findings agree with results recently obtained with guinea pig l ng t se Fredholm and Strandberg 1969) It has previously been shown that phospholipase A has only a very weak histamine releasing effect on isolated peritoneal cells of the rat Keller 1964, Rothschild 1965, Fredholm and Haegermark However lysophosphatides—which can be expected to appear in tissue ex phosphatidase A—have been reported to cause histamine release from var of t ues such as dog liver (Feldberg Holden and Kellaway 1938), guinea F llips and Middleton 1965), rat mast cells (Högberg and Uvnäs 1957 and human leucocytes (Finke, Phillips and Middleton 1965) It has been suggested that the action of phosphatidase A is mediated via the formation of compounds from phosphatides present in the tissue (Feldberg Holden and Kellaway & Trethewie 1939, Rothschild 1965) If this suggestion is correct, the time c the release by F I represents the sum of at least three processes, i.e. diffusion into the tissue, hydrolysis of phosphatides, and lysis of the mast cells As the courses of these processes have not been studied separately no firm conclus n be drawn from the rate of appearance of histamine in the supernatant fluid the shape of the curve obtained in the present work, showing a slow continuous release does not contradict such an idea.

An early described effect of bee venom is excitation of the central nervous system (Langer 1897, Hahn and Ostermayer 1936) This activity has recently been ascribed to a basic polypeptide fraction called apamin (Habermann and Reiz 1964, 1965) In order to assess whether F II had any such activity it was injected into mice in doses of up to ten times the LD_{50} reported for apamin. After the highest dose the animals showed marked symptoms of the anaphylactic type but only slight central nervous system stimulation was produced. Hind leg paralysis similar to that noticed in one of the mice was observed by Hahn and Leditschke (1937) after injecting into mice of a slowly dialysable bee venom fraction, which they called "Komponente II", but to our knowledge this has not been further studied

In conclusion, the results have shown that the two bee venom fractions F I and F II release histamine from rat tissues through different mechanisms The fraction F II, first described by Fredholm (1966) liberates histamine by a mechanism resembling that of *compound 48/80 and thus differs from the earlier described* histamine releasing bee venom fraction melittin. Histamine releasing activity has *been demonstrated for other basic polypeptides such as polymyxin B* (Lagunoff and Benditt 1960), a peptide isolated from *Ascaris suis* (Uvnäs et al 1960, Uvnäs and Wold 1967), and extracts from mammalian tissues (Arthus 1959 and Cyrrus

capillata (Uvnäs 1960) For the *Ascaris* and *Cyanea* polypeptides it has been shown that their action involves energy dependent mechanisms, and it may be that in respect of histamine release F II belongs to this group of compounds Phosphatidase A acts apparently in another way, as its effect is not influenced by agents interfering with metabolic processes This observation is consistent with the assumption that the histamine release caused by phosphatidase A is due to the effect of lyso compounds, resulting from enzymatic breakdown of tissue phosphatides, reactions that do not need metabolic energy

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[illegible] Riksforbundet mot [illegible] of Health U S A [illegible] Jarnros is gratefully acknowledged

References

ARCHER G T The release of histamine from the mast cells of the rat *Aust J exp Biol med Sci* 1959 37 383—390

BREITHAUPT H and E HABERMANN Mastzelldegranulierendes Peptid (MCD Peptid) aus Bienengift Isolierung biochemische und pharmakologische Eigenschaften *Naunyn-Schmiedeberg* [illegible]

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FELDBERG W H F HOLDEN and C H KELLAWAY The formation of lysocithin and of a muscle stimulating substance by snake venoms *J Physiol* (Lond) 1938 *94* 232—248

FELDBERG W and J L MONGAR Comparison of histamine release by compound 48 80 and octylamine in perfused tissues *Brit J Pharmacol* 1954 *9* 197—201

FISKE S R G B PHILLIPS and E MIDDLETON JR Histamine release from human leukocytes by lysolecithin and the effect of *in vitro* anaphylaxis on leukocyte lipids *J Lab clin Med* 1966 *67* 601—609

FREDHOLM B Studies on a mast cell degranulating factor in bee venom *Biochem Pharmacol* 1966 *15* 2037—2043

FREDHOLM B and Ö HAEGERMARK Histamine release from rat mast cells induced by a mast cell degranulating fraction in bee venom. *Acta physiol scand* 1967 *69* 304—312

FREDHOLM B and K STRANDBERG Release of histamine and formation of spasmogenic lipids in guinea pig lung tissue induced by antigen and bee venom phosphatidase A *Acta physiol scand* 1969 *76* 446—457

HABERMANN E Zur Wirkung tierischer Gifte und von Lysocithin auf Grenzflachen *Z ges exp Med* 1958 *130* 19—23

HABERMANN E Recent studies on *Hymenoptera* venoms In *Recent Advances in the Pharmacology of Toxins* (Ed Raudonat) Pergamon Press 1965 pp 53—62

HABERMANN E and K-G REIZ Apamin ein basisches zentral erregendes Polypeptid aus Bienengift *Naturwissenschaften* 1964 *51* 61

HABERMANN E and K-G REIZ Ein neues Verfahren zur Gewinnung der Komponenten von Bienengift insbesondere des zentralwirksamen Peptids Apamin *Biochem Z* 1965 *341* 451—466

HAHN G and H LEDITSCHKE Ueber das Bienengift (IV Mitteilung) Gewinnung beide Giftkomponente durch Dialyse *Ber dtsch chem Ges* 1937 *70* 1637—1644

HAHN G and H OSTERMAYER Ueber das Bienengift (I Mitteilung) *Ber dtsch chem Ges* 1936 *69* 2407—2419

HÖGBERG B and B UVNÄS The mechanism of the disruption of mast cells produced by compound 48/80 *Acta physiol scand* 1957 *41* 345—369

KELLER R. Voraussetzungen fur das Zustandekommen eines zytolysierenden Effektes durch Phosphatidase A. *Helv physiol pharmacol Acta* 1964 *22* C76—C78

LAGUNOFF [illegible]
a new [illegible]

LANGER [illegible]
Pharm [illegible]

MONGAR J and H O SCHILD Inhibition of histamine release in anaphylaxis *Nature* (Lond) 1955 *176* 163

MONGAR J L and H O SCHILD, Effect of temperature on the anaphylactic reaction. *J Physiol* (Lond.) 1957 *135* 320—338

MORAN N C, B UVNÄS and B WESTERHOLM Release of 5-hydroxytryptamine and histamine from rat mast cells. *Acta physiol scand* 1962 *56* 26—41

MOTA I and T ISHII Inhibition of mast cell disruption and histamine release in rat anaphylaxis *in vitro* Comparison with compound 48/80 *Brit J Pharmacol* 1960 *15* 82—87

NEUMANN W and E HABERMANN Beiträge zur Characterisierung der Wirkstoffe des Bienengiftes *Naunyn-Schmiedeberg's Arch exp Path Pharmacol* 1954 *222* 367—387

PERRY W L M, Skin histamine In *Ciba Foundation Symposium on Histamine* J and A. Churchill Ltd. London 1956 pp 242—247

PHILLIPS G B and E. MIDDLETON JR. Release of histamine and slow reacting substance activities from guinea pig lung *Proc Soc exp Biol* (N Y) 1965 *119* 465—470

RILEY J F The mechanism of histamine release from mast cells *J Pharm Pharmacol* 1958 *10* 271—272

ROTHSCHILD A. M Histamine release by bee venom phospholipase A and mellitin in the rat. *Brit J Pharmacol* 1965 *25* 59—66

SHORE P A A. BURKHALTER and V H COHN, JR. A method for the fluorometric assay of histamine in tissues *J Pharmacol* 1959 *127* 182—186

TREHERNE J E The permeability of skin to some non-electrolytes *J Physiol* (Lond.) 1956 *133* 171—180

TRETHEWIE E R. Comparison of haemolysis and liberation of histamine by two Australian snake venoms *Aust J exp Biol med Sci* 1939 *17* 145—155

UVNÄS B Mechanism of action of a histamine liberating principle in jellyfish *(Cyanea capillata* Ann N Y *Acad Sci* 1960 *90* 751—759

[illegible] 1 3 137—149

[illegible] mast cell disruption in [illegible] 1960 *199* 575—578

[illegible] polypeptide from *Ascaris* [illegible]

YAMASAKI H and K ENDO Coexistence of energy dependent and non-dependent processes in histamine release mechanism of compound 48/80 and sinomenine experiments on chopped skin from various animal species *Jap J Pharmacol* 1967 *17* 228—239

ZEYA H J and J K SPITZNAGEL Cationic proteins of polymorphonuclear leukocyte lysosomes. *J Bact* 1966 *91* 750—754

Acta physiol. scand. 1969. 76. 299—311
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Metabolism of Dopamine and Noradrenaline in Normal, Atrophied and Postganglionically Sympathectomized Rat Salivary Glands in vitro

By

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Received 13 September 1968

Abstract

JONASON, J *Metabolism of dopamine and noradrenaline in normal, atrophied and postganglionically sympathectomized rat salivary glands in vitro* Acta physiol scand 1969 *76* 299—311

Slices from normal atrophied and postganglionically sympathectomized rat salivary glands were incubated with labeled dopamine or noradrenaline The catabolism of dopamine proceeded at a higher rate than that of noradrenaline This phenomenon is proposed to be due to the higher affinity of dopamine for monoamine oxidase the extraneuronal part of which being very important The major catabolites of dopamine were found to be the phenolic acids dihydroxyphenylacetic acid and homovanillic acid Exogenously added noradrenaline was primarily catabolized to normetanephrine whereas the major metabolites of newly synthesized noradrenaline from dopamine were the phenolic glycols 3 4-dihydroxyphenylglycol and 3 methoxy-4 hydroxyphenylglycol, supporting the view that intraneuronally formed noradrenaline initially is metabolized by intraneuronal monoamine oxidase prior to its O methylation by catechol O methyl transferase Atrophy reduced the levels of all 3-O methylated products especially those of the methoxylated amines whereas sympathectomy increased the amounts of these amines and diminished the deaminated products Thus catechol O methyl transferase is localized to the parenchymal cells in the salivary glands. In contrast to the dopamine metabolism the catabolism of noradrenaline was more affected by postganglionical sympathectomy than by atrophy The data from the present study demonstrate the importance of the amine concentrating mechanism of the adrenergic nerve cell membranes.

The metabolism of dopamine (DA) and noradrenaline (NA) in both the central and peripheral nervous system has been extensively studied during the last decade (for review see *e g* Axelrod 1965 Glowinski and Baldessarini 1966 Iversen 1967) The enzymes monoamine oxidase (MAO) and catechol O methyl transferase (COMT are of great importance in the catabolism of these catecholamines (Axelrod *et al* 1958 Axelrod Albers and Clementi 1959, Rosengren 1960, Crout Creveling and Udenfriend 1961 La Brosse *et al* 1961 Carlsson and Hillarp 1962 Mannarino Kirshner and Nashold 1963 Glowinski Kopin and Axelrod 1965) It is also well known that dopamine β hydroxylase (the enzyme converting DA to NA) is important in the metabolism of DA (Udenfriend and Creveling 1959, Carlsson 1959 Kirshner 1959 Potter and Axelrod 1963 Kaufmann and Friedmann 1965, Glo-

winski, Axelrod and Iversen 1966, Merritt and Schultz 1966) In sympathetically innervated organs this enzyme is considered to be localized within the storage granules which are located within the adrenergic neuron (Potter and Axelrod 1963, Iversen Glowinski and Axelrod 1966) The activity of COMT is not affected by sympathectomy (Waltman and Sears 1964, Potter *et al* 1965, Iversen *et al* 1966) Thus COMT is thought to be localized outside the neuron in both peripheral tissues (Kopin and Gordon 1962) and brain (Carlsson and Hillarp 1962 Jonason and Rutledge 1968a) MAO, on the other hand, is localized both within the neuron and outside the neuron in sympathetically innervated tissues (Snyder, Fischer and Axelrod 1965 Almgren *et al* 1966) In fact, it has been demonstrated that in rat salivary glands about one third of the MAO activity is localized within the adrenergic nerves and about two thirds within the parenchymal cells (Almgren *et al* 1966)

The salivary glands have many advantages in studying intraneuronal versus extraneuronal metabolism of catecholamines By extirpation of the superior cervical ganglion this organ can be postganglionically sympathectomized and extraneuronal metabolism can be studied Furthermore, it has been reported that the salivary gland cells atrophy after ligation of the excretory ducts (Junqueira 1951, Bhaskar, Bolden and Weinmann 1956, Standish and Shafer 1957) This atrophy is not accompanied by a corresponding loss of NA (Anden, Norberg and Olson 1966) The MAO activity in such atrophied glands are reduced by about two thirds (Almgren *et al* 1966) The NA reduction rate after reserpine is not affected by this operation but after pretreatment with the MAO inhibitor nialamide reserpine produced a slower decrease in the atrophied glands than in the intact ones (Jonason 1968) This phenomenon might be due to loss of COMT activity if this enzyme is localized within or at the surface of the parenchymal cells Obviously after reserpine treatment the intraneuronal part of MAO is responsible for the metabolism of most of the NA Atrophied salivary glands seem to be a useful tool for investigations of intraneuronal metabolism of catecholamines

Recently the metabolic pathways of DA and NA in rabbit brain have been investigated using slice technique (Rutledge and Jonason 1967) It was found that DA was deaminated to the two phenolic acids 3 4 dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) whereas NA was deaminated primarily to the two phenolic glycols 3 methoxy-4 hydroxyphenylglycol (MOPEG) and 3 4 dihydroxyphenylglycol (DOPEG) The same phenomenon has been found in rabbit atria (Rutledge and Weiner 1967) These findings have prompted the present study on the metabolism of DA and NA in normal atrophied and sympathetically denervated salivary glands in order to gain informations of normal metabolism of catecholamines intra *versus* extraneuronal metabolism of these monoamines and the localization of the involved enzymes in these glands

Material and methods

Adult male Sprague Dawley rats weighing about 200 g were used In one group of animals the excretory ducts of the left submaxillary and sublingual glands were ligated near the hilum In another group the submaxillary and sublingual glands were postganglionically sympathec

All the operations were the operation e used Before observing the

roform gland id was glands unt of ml of Krebs-Henseleit solution for 10 min at 37° in 95% O_2-5% CO_2 (Rutledge and Weiner 1967) Then 5670×10^{-12} moles of C^{14} DA (46.5×10^{-9} curies) or H^3 NA (6.66×10^{-7} curies) were added and the flasks were incubated an additional 20 min Control samples were prepared by the addition of 2 ml 2 N HCl to the flasks before the preincubation period All incubations were terminated by the addition of 2 ml 2 N HCl

When the retention of the amines was measured the slices were blotted and dried upon filter paper after the 20 min incubation and incubated an additional 10 min in a substrate free medium The slices were blotted and dried again and the total radioactivity retained by the slices was determined

Procedures in this study have been described in detail in previous publications (Rutledge and Jonason 1967, Jonason and Rutledge 1968a) and thus will be presented only in general terms DA and NA were isolated by adsorption onto a Dowex 50 column Methoxytyramine (MTA) and normetanephrine (NM) were first passed over alumina where the two catechol amines DA and NA were adsorbed The alumina effluent containing MTA and NM, was then passed over a Dowex 50 column and the two amines were eluted in separate fractions When the deaminated catabolites were determined the Dowex effluent was passed over ... andelic acid nylglycol hydroxy nylglycol MA) was and the volumes were reduced for either paper or thin layer chromatography The compounds were eluted from the chromatograms with 0.2 N acetic acid Recoveries were determined in each sample by adding carrier substances immediately upon termination of the incubation and quantification of the amount remaining in the eluate after the separation procedures The amount of amines recovered was measured by the fluorescence emitted at 335 mμ upon activation at 280 mμ The amount of deaminated catabolites was quantitated by the phenol reaction of Barness *et al* (1963) The radioactivity of each of the amines and catabolites was determined by liquid scintillation counting technique in a Packard Tricarb scintillation apparatus after freeze-drying

The ... a were These specific e same manner with the exception that no recovery values could be assessed thus no correction for this variable was made It was assumed that the recoveries from this group of compounds were relatively constant Results are generally expressed as mean ± standard error of the mean (S E M

Results

Metabolism of exogenously added DA in normal, atrophied and sympathectomized salivary glands

The results are presented in Table I The rate of metabolism of DA in normal salivary gland slices was high After 20 min incubation 28 per cent of the substrate was unmetabolized (compared to the control values) The major metabolites of DA were (listed in order of quantitative importance) DOPAC, HVA, MTA and NA The phenolic alcohols DOPET and MOPET were of no quantitative importance.

This is in good agreement with the conditions in rabbit brain cortex (Rutledge and Jonason 1967 Jonason and Rutledge 1968a) and in the rabbit atria (Rutledge and Weiner 1967) However, in the rabbit brain cortex the metabolic rate of DA was found to be somewhat slower and DOPAC seemed to be quantitatively a more dominating metabolite than in the salivary glands In these glands metabolism of exogenously added DA by COMT appeared to be of greater quantitative importance than in rabbit brain cortex

After atrophy of the parenchymal cells the metabolic rate of DA was significantly decreased 59 per cent of added DA was unmetabolized compared to the control values (P<0 001) All 3 O methylated compounds were diminished MTA 16 per cent HVA 39 per cent and MOPET 50 per cent of the normal values respectively (after subtracting the control values) The dihydroxy compound DOPAC was slightly reduced whereas the small value of DOPET actually was slightly increased It is obvious that the COMT activity is severely decreased after atrophy The relatively large control values of NA are due to partial contamination from the C^{14} DA precursor (1 3 per cent of the substrate) It might be surprising to find that after sympathectomy the NA values were less than 50 per cent of the control values However in this case the level of C^{14} DA was much lower than in the control Actually the NA values after sympathectomy are in good agreement with the control values (about 1 3 per cent of the C^{14} DA)

Sympathetic denervation of the salivary glands produced a slightly reduced metabolic rate of DA (40 per cent of added DA unmetabolized) The most striking effect of this operation was the three fold increase in the amount of MTA (P<0 001) whereas the level of the corresponding acid HVA was reduced (39 per cent of normal) The low level of MOPET was not affected The dihydroxy compounds DOPAC and DOPET were reduced NA was not found after sympathectomy

Metabolism of newly formed NA in normal atrophied and sympathectomized salivary glands

NA was synthetized from DA by rat salivary gland slices (Table I) The most important metabolites of this newly formed NA from a quantitative point of view were the glycols DOPEG and MOPEG NM DOMA and VMA were found only in small quantities. This is consistent with results from rabbit brain cortex (Rutledge and Jonason 1967 Jonason and Rutledge 1968a) Atrophy did not affect the synthesis of NA from DA whereas sympathectomy reduced the level of NA to zero The small values of the individual metabolites do not allow to make any certain conclusions of the effects of atrophy and sympathectomy but it is obvious that MOPEG after atrophy was markedly reduced (7 % of normal values VMA and DOMA could not be detected after atrophy whereas NM and DOPEG were not significantly affected Sympathectomy seemed to reduce all the metabolites except the small amounts of NM It appears from Table I as if some DOPEG actually was formed from newly synthetized NA from DA However the values were rather small The reason for this phenomenon is not known It might be an incomplete separation between DO

TABLE I Metabolism of dopamine in rat salivary gland slices

Slices from normal, atrophied and postganglionically sympathectomized rat salivary glands were incubated for 20 min with 5670 × 10 $^{-11}$ moles (46 5 × 10 $^{-9}$ curies) of C^{14} dopamine Control corresponds to samples in which 2 ml 2 N HCl were added to the incubation fluid before the incubation Mean recoveries of the metabolites based upon 19—52 values dopamine (DA) 91 0 % ± 1 8 methoxytyramine (MTA) 73 2 % ± 2 1, 3 4-dihydroxyphenylacetic acid (DOPAC) 44 2 % ± 1 6, homovanillic acid (HVA) 64 7 % ± 3 4, 3,4-dihydroxyphenylethanol (DOPET) 45 2 % ± 1.9 3 methoxy 4 hydroxyphenylethanol (MOPET) 23 5 % ± 2 2, noradrenaline (NA) 92 5 % ± 1 6, normetanephrine (NM) 80 6 % ± 0 7, 3 4-dihydroxymandelic acid (DOMA) 15 3 % ± 0 9, 3 methoxy 4-hydroxymandelic acid (VMA) 14 0 % ± 1 3, 3,4-dihydroxyphenyl glycol (DOPEG) 16 4 % ± 1 0 3 methoxy 4 hydroxyphenylglycol (MOPEG) 34 0 % ± 2 3 All values were corrected for recovery n represents the number of experiments S E. M represents the standard error of the mean The values are presented as moles × 10 $^{-11}$ and represent the amounts of the compounds in the media plus that in the tissue Values in parenthesis represent values in which the control values have been subtracted * significantly different from normal values at P < 0 05, *** P < 0 001

		Normal glands	Atrophied glands	Sympathectomized glands	Control
DA	Mean	1470	3040***	2051*	5180
	S E. M	183	145	169	533
	n	12	7	10	5
MTA	Mean	213 (196)	48 8*** (32 0)	607*** (590)	16 8
	S E M	7 7	4 5	33 8	0 5
	n	6	5	4	4
DOPAC	Mean	529 (526)	393* (390)	210*** (207)	2 5
	S E M	33 6	35 4	13 6	0 1
	n	8	6	5	3
HVA	Mean	476	186***	362***	0 6
	S E M	57 5	20 7	40 9	0 6
	n	7	5	4	3
DOPET	Mean	35 2	45 1***	17 8***	0 6
	S E M	3 1	5 5	2 1	0 4
	n	9	6	5	3
MOPET	Mean	20 8	10 5***	25 6	0 3
	S E M	4 6	3 8	3 8	0 3
	n	7	6	4	3
NA	Mean	239	269	29 3***	65 5
		173	(203)	(0)	
	S E M	19 4	29 8	10 2	19 2
	n	6	4	5	4

Table I (Continued)

		Normal glands	Atrophied glands	Sympathectomized glands	Control
NM	Mean	23.5 (5.0)	25.0 (6.5)	37.4 (18.9)	18.5
	S.E.M.	3.1	2.8	5.1	2.3
	n	6	5	4	4
DOMA	Mean	21.6 (9.2)	6.5 (0)	1.0 (0)	12.4
	S.E.M.	12.4	2.4	0.8	12.4
	n	6	6	4	3
VMA	Mean	20.4 (18.3)	0	13.8 (11.7)	2.1
	S.E.M.	2.8	0	7.7	2.1
	n	7	4	3	3
DOPEG	Mean	158 (153)	122 (117)	108 (103)	4.6
	S.E.M.	35.0	35.8	23.4	3.6
	n	9	6	5	2
MOPEG	Mean	120	8.0	14.1	0.9
	S.E.M.	63.2	3.3	4.6	0.9
	n	4	4	2	3

PAC and DOPEG in the chromatograms. The existence of a "site" (not localized to the adrenergic neurons) which has the capacity of metabolizing DA to DOPEG cannot be excluded.

Metabolism of exogenously added NA in normal, atrophied and sympathectomized salivary glands

The results are presented in Table II. NA was metabolized at a slower rate than DA. Normally 64 per cent of added NA was unmetabolized after 20 min incubation (calculated by comparing the experimental values to the control values). This is in good agreement with data from rabbit brain *in vitro* (Rutledge and Jonason 1967, Jonason and Rutledge 1968 a, b). In contrast to the conditions in rabbit brain the glycols of NA were not quantitatively the major catabolites of exogenously added NA in the salivary glands. The catabolites of NA in these glands were (listed in order of quantitative importance): NM (which constitutes about 60 per cent of total catabolites), DOPEG, VMA, MOPEG and DOMA. The basic difference between these data and those of rabbit brain cortex is that 3-O-methylation by COMT appears to play a greater role in the salivary glands after exogenous administration of NA. The levels of NM and VMA were higher compared to the brain cortex while

TABLE II Metabolism of exogenously added noradrenaline in rat salivary gland slices

Slices from normal atrophied and postganglionically sympathectomized rat salivary glands were incubated for 20 min with 5670 × 10 $^{-12}$ moles (6 66 × 10 $^{-7}$ curies) of H^3 noradrenaline For further explanation see Table I (abbreviations, recoveries etc) All values were corrected for recovery and are expressed as moles × 10 $^{-12}$ The values represent the amounts of the compounds in the media plus that in the tissue Values in parenthesis represent values in which the control values have been substracted * significantly different from normal values at P < 0 05, ** P < 0 01, *** P < 0 001

		Normal glands	Atrophied glands	Sympathecto-mized glands	Control
NA	Mean	3071	3614	3857*	4871
	S E M	126	136	310	213
	n	9	10	4	6
NM	Mean	759 (707)	111*** (59)	1269*** (1217)	51 6
	S E M	53 7	26 0	42 6	9 0
	n	8	7	5	4
DOMA	Mean	63 4 (59 3)	83 6 (79 5)	20 4*** (16 3)	4 1
	S E M	4 4	13 0	4 1	2 1
	n	8	11	3	6
VMA	Mean	112 (106)	56 3* (50 4)	73 8* (67 9)	5 9
	S E M	12 0	12 9	5 4	1 3
	n	5	5	4	4
DOPEG	Mean	210 (205)	355** (350)	52 1** (46 7)	5 4
	S E M	25 7	29 2	2 3	3 6
	n	7	11	4	4
MOPEG	Mean	107 (97 8)	74 6* (65 4)	113 (104)	9 2
	S E M	10 6	4 7	18 6	2 1
	n	13	11	8	5

especially the levels of DOMA DOPEG and even MOPEG were lower The relatively large control values of NM are due to partial contamination from the H^3 NA precursor

After atrophy there was no significant increase of unmetabolized NA The level of all 3 O methylated products were decreased while the dihydroxy compounds either were increased or unchanged NM constituted only 8 per cent of the normal values (P<0 001) The corresponding values for VMA and MOPEG were 47 and 67 per cent, respectively (P<0 05) The level of DOPEG was increased (P<0 01) whereas the values of DOMA were not significantly elevated compared to the normal ones

TABLE III Retention of dopamine and noradrenaline in rat salivary gland slices

Slices from normal, atrophied and postganglionically sympathectomized rat salivary glands were incubated for 20 min with 5670 × 10 moles of either C^{14} dopamine (46.5 × 10 curies) or H^{3} noradrenaline (6.66 × 10 curies). The values represent the amount of amine (or metabolic product) retained by the slices after a 10 min postincubation in a substrate free medium and are expressed as moles of amine × 10.
*** Significantly different from normal values at $P < 0.001$

		Normal glands	Atrophied glands	Sympathectomized glands	Control
DA	Mean	418	387	180***	16.7
		(401)	(370)	(163)	
	S.E.M.	38.3	42.4	20.1	3.9
	n	11	5	8	6
NA	Mean	479	433	189***	6.4
		(473)	(427)	(183)	
	S.E.M.	27.5	43.0	7.2	0.9
	n	6	5	5	4

Sympathectomy produced a decreased rate of metabolism of NA. 79 per cent of added NA was unmetabolized after 20 min incubation ($P<0.05$). The level of NM was significantly increased ($P<0.001$) whereas markedly decreased levels of the dihydroxy compounds DOMA and DOPEG were observed. The amount of VMA also was slightly lowered while that of MOPEG was unchanged.

Retention of DA and NA in normal, atrophied and sympathectomized salivary glands

From Table III it can be seen that normally about equal amounts of DA and NA were retained by the slices. The amounts retained appear to be about the same as in the caudate nucleus of rabbits (Jonason and Rutledge 1968b), but somewhat less than the amounts retained by rabbit brain cortex slices (Jonason and Rutledge 1968a). Atrophy of the parenchymal cells of the salivary glands did not significantly reduce the retention ability of the slices. However, after sympathectomy there was a marked decrease of retained amines (or catabolites) to about one third of the normal values for both of the amines ($P<0.001$).

Overall recoveries of amines and metabolites

If the amounts of the substrate and all the metabolites are summed and this value are compared to the control values of the substrate it is found that the recovery is good when NA is used as substrate (between 87 and 106 per cent). When the corresponding comparison for DA is made, the recovery values are found to be between 62 and 78 per cent. The reason for this discrepancy is not known. It might partly be due to autoradiolysis during the time consuming separation procedure. The existence

of other metabolites which were not analyzed e g oxidation products (Axelrod 1964) or conjugates cannot be excluded The major conclusions drawn in this study is not affected by this somewhat low recovery

Discussion

It is evident from this study that the rate of metabolism of catecholamines in normal salivary gland slices is high After a 20 min incubation period only 28 per cent of added DA and 64 per cent of added NA were unmetabolized The corresponding values of rabbit brain cortex cerebri slices were about 50 and 79 per cent respectively after 30 min incubation, using about equal amounts of substrate and tissue (Rutledge and Jonason 1967) This difference in metabolic rate between rabbit brain and rat salivary glands is probably due to different metabolic properties in the two tissues but species differences cannot be excluded However just as in rabbit brain cortex slices DA is metabolized at a faster rate than NA in salivary gland slices

The general pattern of the DA metabolites appears to be about the same in rat salivary glands and rabbit brain cortex (Rutledge and Jonason 1967) However DOPAC does not seem to be as predominant in these glands as in rabbit brain cortex but the deaminated metabolites of DA are primarily phenolic acids as was found in rabbit brain tissues (Rutledge and Jonason 1967 Jonason and Rutledge 1968b) The same investigators also found that the predominant metabolites of NA in these tissues are the phenolic glycols a phenomenon which earlier has been reported from rabbit atria (Rutledge and Weiner 1967) As seen from this study (Table II) this is not true for the salivary glands NM is in this organ the major metabolite of exogenously added NA and constitutes about 60 per cent of the total catabolites O methylation by COMT appears to play a very important role in the metabolism of NA in the salivary glands in contrast to *e g* the caudate nuclei *in vitro* (Jonason and Rutledge 1968b) It is also known that the salivary glands have a particularly high activity of COMT (Axelrod Albers and Clementi 1959) The glycols DOPEG and MOPEG constitute together about 26 per cent of the total catabolites whereas the corresponding value of VMA plus DOMA is about 14 per cent

There is an interesting difference between the metabolism of exogenously added NA and the metabolism of newly synthesized NA from DA Newly formed NA is metabolized primarily to the phenolic glycols DOPEG and MOPEG the other metabolites being formed only in small quantities (Table I) Thus the level of NM is low This phenomenon supports the view that intraneuronally formed NA is initially deaminated by intraneuronal MAO prior to its O methylation by extraneuronal COMT Carlsson and Hillarp 1962 Kopin and Gordon 1962)

It is known that about one third of the MAO activity is localized intraneuronally and about two thirds outside the neurons in the salivary glands (Almgren *et al* 1966) The extraneuronal part of the enzyme is localized in the parenchymal cells of the glands The enzyme COMT on the other hand is thought to be localized outside the adrenergic neurons Kopin and Gordon 1962 Carlsson and Hillarp 1962

Waltman and Sears 1964 Potter *et al* 1965, Iversen *et al* 1966 Jonason and Rutledge 1968a) The evidence for this view are of more or less indirect nature The present study shows that after atrophy of the parenchymal cells of the salivary glands there is a severe decrease of COMT activity (Table I and II) All 3 O methylated products are diminished especially the methoxylated amines NM constitutes only 8 per cent of the normal value using NA as substrate whereas the corresponding value of MTA using DA as substrate is 16 per cent After postganglionical sympathectomy of the glands those products which are only 3 O methylated namely MTA and NM, are markedly increased those products which are both 3 O methylated and deaminated (HVA VMA, MOPET and MOPEG) are either reduced or unchanged whereas those products which are only deaminated (DOPAC DOMA DOPET and DOPEG) are reduced These data strongly indicate that the enzyme COMT to the greatest extent is localized within or at the surface of the parenchymal cells of the salivary glands

It is obvious from this investigation that normally DA is metabolized at a faster rate than NA in the salivary glands The same phenomenon is observed after sympathectomy (Table I and II) Thus the reason for this difference in metabolic rate between DA and NA might be unequalities in extraneuronal metabolism of these amines The affinities of these amines for COMT are about the same (Axelrod and Tomchick 1958) Furthermore the level of NM using NA as substrate is higher than that of MTA from DA (Table I and II) However, DA is known to be a better substrate for MAO than NA (Blaschko Richter and Schlossman 1937 Weiner 1960) Thus DA is probably metabolized by extraneuronal and intraneuronal MAO to a greater extent than NA The total sum of deaminated metabolites of DA is diminished about equally by atrophy and sympathetic denervation whereas that of NA is not affected by atrophy but decreased after sympathetic denervation These data indicate that extraneuronal MAO plays a greater role for inactivation of DA than that of NA Atrophy which results in severe loss of both MAO (Almgren *et al* 1966) and COMT (as seen from this investigation) has a more pronounced effect on the metabolic rate of DA than sympathetic denervation The reason for this phenomenon seems primarily to be the loss of MAO activity by this procedure Thus, the data from this investigation indicate that DA is metabolized at a faster rate than NA in salivary gland slices mostly because of its higher affinity for MAO the extraneuronal part of this enzyme being very important

Reduction of the MAO activity by atrophy does not diminish the level of the direct deaminated product of DA namely DOPAC proportionally In fact when NA was used as substrate the direct deaminated products namely DOPEG and DOMA were actually increased or unaltered (Table II) It could be argued that this phenomenon is due to unability of further catabolism by COMT to form MOPEG and VMA However, the increase in amounts of DOPEG and DOMA is about twice of the decrease in the levels of MOPEG and VMA after atrophy Furthermore NA reduction rate after reserpine is not affected by atrophy in spite of the severe loss of both MAO and COMT activities (Jonason 1968) It is probable that the intraneuronal

part of MAO is highly important in the metabolism of NA because of the ability of the adrenergic nerves to concentrate the substrate inside the nerve by the membrane pump mechanism (Hamberger and Masuoka 1965 Glowinski and Axelrod 1966 Carlsson *et al* 1966 Haggendal and Hamberger 1967) The substrate concentration for intraneuronal MAO and the "membrane pump mechanism is increased by atrophy This situation could explain why the deamination reactions of NA are not so much affected by reducing the MAO activity in the glands by two thirds Probably the membrane pump very effectively concentrates the amines inside the neurons This view is supported by the fact that the levels of the 3 O methylated amines MTA and NM are increased after sympathetic denervation of the glands (Table I and II) In normal glands the enzyme COMT is probably exposed to lower substrate concentrations than in the sympathectomized glands because of the effectiveness of the membrane pump which results in lower levels of MTA and NM It is not unlikely that NA utilizes the membrane pump to a greater extent than DA (Jonason and Rutledge 1968a) A support for this view is that atrophy did not significantly reduce the metabolic rate of NA in contrast to that of DA whereas postganglionical sympathectomy had a greater effect than atrophy on the NA metabolism (also in contrast to DA metabolism) If NA is a better substrate than DA for the amine concentrating mechanism at the level of the cell membrane of the adrenergic nerves sympathectomy would be expected to affect NA metabolism to a greater extent than DA metabolism

Normally about equal amounts of DA and NA were retained by the salivary gland slices (Table III) Protriptyline pretreatment of the animals reduced the retention of NA to 18 per cent of normal whereas the retention of DA was diminished to 30 per cent of normal using the same substrate concentration and amount of slices as in this investigation (Jonason 1968—to be published) The retention ability of the salivary gland slices was reduced to about one third of normal for both DA and NA by sympathectomy Thus protriptyline might have some extraneuronal effects in the metabolism of NA Atrophy did not significantly reduce the retention ability of the slices Loss of possible extraneuronal binding sites in the parenchymal cells (Almgren Anden and Waldeck 1965) does not seem to affect the ability of retention of catecholamines by the salivary gland slices

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References

ALMGREN O N E ANDEN J JONASON K A NORBERG and L OLSON Cellular localization of monoamine oxidase in rat salivary glands *Acta physiol scand* 1966 67 21—26

ALMGREN O N E ANDEN and B WALDECK Extraneuronal binding as a possible factor of t ram ... 6

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AXELROD, J, The metabolism, storage and release of catecholamines *Rec. Progr Hormone Res* 1965 *21* 597—622

AXELROD, J, W. ALBERS and C D CLEMENTI, Distribution of catechol O methyl transferase in the nervous system and other tissues *J Neurochem* 1959 *5* 68—72

AXELROD, J, J K INSCOE, S SENOH and B WITKOP, O-methylation, the principal pathway for the metabolism of epinephrine and norepinephrine in the rat *Biochim biophys Acta* (Amst) 1958 *27* 210—211

AXELROD, J and R TOMCHICK, Enzymatic O methylation of epinephrine and other catechols *J biol Chem* 1958 *233* 702—705

BARNESS L A, W J MELLMAN, T TEDESCO, D G YOUNG and R NOCHO, A quantitative method of determining urinary phenols *Clin Chem* 1963 *9* 600—607

BHASKAR S N, T E BOLDEN and J P WEINMANN, Experimental obstructive adenitis in the mouse *J dent Res* 1956 *35* 852—862

BLASCHKO H, H D RICHTER and H SCHLOSSMANN, The oxidation of adrenaline and other amines *Biochem J* 1937 *31* 2187—2198

CARLSSON, A The occurrence distribution and physiological role of catecholamines in the nervous system *Pharmacol Rev* 1959 *11* 490—493

CARLSSON A, K FUXE B HAMBERGER and M LINDQVIST, Biochemical and histochemical studies on the effects of imipramine like drugs and (+)-amphetamine on central and peripheral catecholamine neurons *Acta physiol scand* 1966 *67* 481—497

CARLSSON A and N-A HILLARP, Formation of phenolic acids in brain after administration of 3 4-dihydroxyphenylalanine *Acta physiol scand* 1962 *55* 95—100

CROUT J R C R CREVELING and S UDENFRIEND Norepinephrine metabolism in rat brain and heart *J Pharmacol exp Ther* 1961 *132* 269—277

GLOWINSKI J and J AXELROD, Effects of drugs on the disposition of H^3-norepinephrine in the rat brain *Pharmacol Rev* 1966 *18* 775—785

GLOWINSKI J J AXELROD and L L IVERSEN, Regional studies of catecholamines in the rat brain IV Effects of drugs on the disposition and metabolism of H^3 norepinephrine and H^3-dopamine *J Pharmacol exp Ther* 1966 *153* 30—41

GLOWINSKI J and R J BALDESSARINI, Metabolism of norepinephrine in the central nervous system *Pharmacol Rev* 1966 *18* 1201—1238

GLOWINSKI J I J KOPIN and J AXELROD, Metabolism of H^3 NE in the rat brain *J Neurochem* 1965 *12* 25—30

HAGGENDAL J and B HAMBERGER, Quantitative in vitro studies on noradrenaline uptake and its inhibition by amphetamine, desipramine and chlorpromazine *Acta physiol scand* 1967 *70* 277—280

HAMBERGER B and D MASUOKA Localization of catecholamine uptake in rat brain slices *Acta pharmacol* (Kbh) 1965 *22* 363—368

IVERSEN, L L *The Uptake and Storage of Noradrenaline in Sympathetic Nerves* Cambridge University Press, Great Britain 1967

IVERSEN L L J GLOWINSKI and J AXELROD The physiologic disposition and metabolism of norepinephrine in immunosympathectomized animals *J Pharmacol exp Ther* 1966 *151* 273—284

JONASON, J Effects of reserpine on the noradrenaline content of atrophied rat salivary glands *Acta physiol scand* 1969 *75* 73—77

JONASON, J and C O RUTLEDGE, The effect of protriptyline on the metabolism of dopamine

-d 1968a *73* 161 175

e and noradrenaline in rat b

-417

observations on secreting and

macol Rev 1965 *17* 71 100

harmacol Rev 1959 *11* 3 0

360

KOPIN, I J and E K GORDON Metabolism of norepinephrine H^3 released by tyramine and reserpine *J Pharmacol exp Ther* 1962 *138* 351—359

LABROSSE, E H, J AXELROD I J KOPIN and S S KETY Metabolism of 7 H^3-epinephrine d bitartrate in normal young men *J clin Invest* 1961 *40* 253 260

MANNARINO, E, N KIRSHNER and B S NASHOLD The metabolism of C^{14} noradrenaline by cat brain in vivo *J Neurochem* 1963 *10* 373—379

MERRITT, J H and E J SCHULTZ, The effect of decarborane on the biosynthesis and metabolism of norepinephrine in the rat brain *Life Sci* 1966 *5* 27—32

POTTER L T and J AXELROD Properties of norepinephrine storage particles of the rat heart *J Pharmacol exp Ther* 1963 *142* 299—305

POTTER L T, T COOPER, V L WILLMAN and D E WOLFE, Synthesis, binding, release and metabolism of norepinephrine in normal and transplanted dog heart *Circulat Res* 1965 *16* 468—481

ROSENGREN, E, On the role of monoamine oxidase for the inactivation of dopamine in brain *Acta physiol scand* 1960 *49* 370—375

RUTLEDGE C O and J JONASON, Metabolic pathways of dopamine and norepinephrine in rabbit brain *in vitro* *J Pharmacol exp Ther* 1967 *157* 493—502

RUTLEDGE, C O and N WEINER The effect of reserpine upon the synthesis of norepinephrine [illegible]

[illegible] ne oxidase

[illegible] sublingual

[illegible] *J Neuro-chem* 1959 *4* 350—352

WALTMAN, S and M SEARS, Catechol O methyl transferase and monoamine oxidase activity in the ocular tissues of albino rats *Invest Ophtal* 1964 *3* 601—605

WEINER N Substrate specificity of brain monoamine oxidase of several mammals *Arch Biochem* 1960 *91* 182—188

Acta physiol. scand. 1969. 76. 312—320
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Enzyme Activities at the Surface of Intact Human Erythrocytes

By

GUNNAR RONQUIST

Received 16 September 1968

Abstract

RONQUIST, G *Enzyme activities at the surface of intact human erythrocytes* Acta physiol scand 1969 *76* 312—320

The activity of glyceradehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and adenylate kinase was determined at the surface of intact red cells, suspended in isotonic sodium chloride or isotonic sodium chloride containing 6 % Dextran The surface-bound phosphoglycerate kinase activity was found to be 4 5 % of the total phosphoglycerate kinase activity of the cell The corresponding figures for glyceraldehyde-3-phosphate dehydrogenase and adenylate kinase were 3 1 % and 2 0 %, respectively The extent of hemoglobin leakage into the assay medium did not exceed 1 % in any case The suspending medium, after the cells had been removed contained no glyceraldehyde 3-phosphate dehydrogenase or phosphoglycerate kinase activity, whereas part of the adenylate kinase activity had been eluted from the cells

The formation of extracellular (^{32}P)adenosine triphosphate by human erythrocytes incubated with (^{32}P)orthophosphate and the phosphorylated substrates and cofactors of glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate NAD oxidoreductase (phosphorylating), EC 1 2 1 12) and phosphoglycerate kinase (ATP D-3-phosphoglycerate 1-phosphotransferase EC 2 7 2 3) was studied in a previous work (Ronquist 1968) Evidence was obtained that most of the extracellular (^{32}P)ATP had been formed by enzymes bound to the outer surface of the red cells It was therefore of interest to determine the activity of each single enzyme possibly concerned

Thus, the present investigation deals with the determination of glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and adenylate kinase (ATP AMP phosphotransferase, EC 2 7 4 3) at the red cell surface, as well as that in a total hemolysate Various other enzymes of the red cell were likewise tested for activity at the surface and in the hemolysate

Material and methods

Special chemicals

AMP, ADP, ATP, NADH NADP$^+$, 3 phosphoglycerate (sodium salt) phosphoenolpyruvate (tricyclohexylammonium salt) 2 3 diphosphoglycerate (pentacyclohexylammonium salt) fructose 1,6 diphosphate (sodium salt), fructose 6 phosphate (barium salt), 2 phosphoglycerate

(barium salt), glutathione (oxidized form), glucose 6 phosphate (sodium salt) pyruvate (sodium salt) and all enzymes were purchased from Boehringer & Soehne GmbH Mannheim, Germany The 2 phosphoglycerate and fructose 6 phosphate were rendered Ba^{++} free before use by making them weakly acid and precipitating the barium ions with potassium sulfate

Analytical methods

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Hematocrit was determined in an Adams readacrit centrifuge Es 75M-4 6? (Clay Adams New York 10 N Y) Osmolarity was measured in a Vapor Pressure Osmometer Model 301 A Mechrolab, Hewlett & Packard (by courtesy of Dr R Stromberg AB Pharmacia Uppsala Sweden)

Preparation of red cells

Venous blood samples from healthy individuals were collected in heparinized tubes Only freshly drawn blood was used throughout the experiments The blood was centrifuged at $1800 \times g$ for 6 min, and the plasma and the buffy coat layer were removed by suction The red cells were washed 4 times at 10—15° C with 0 15 *M* sodium chloride and centrifuged at $1800 \times g$ for 6 min When red cells were suspended in 0 15 *M* NaCl containing 6 % (w/v) Dextran (Dextran T 40 $M_w = 41\,800$ $M_n = 25\,700$ $/\eta/ - 0\,210$, AB Pharmacia Uppsala Sweden) the third and fourth washings were performed with this solution The red cells were used immediately after preparation

Preparation of hemolysate

Hemolysates were prepared by freezing and thawing an aqueous suspension of washed red cells (red cells diluted 10—60 times with distilled water) The enzymes of the hemolysate were either measured directly or within one week, being kept frozen in the meantime

Enzyme assays

All enzymatic analyses were performed in an assay medium buffered with triethanolamine hydrochloric acid buffer, pH 7 6 0 05 *M* with respect to total triethanolamine In the experiments using red cells suspended in sodium chloride Dextran the buffer contained 6 % Dextran

Phosphofructokinase was determined as described by Vogell *et al* (1959 For all other enzymes tested the methods given in the Biochemica Information Boehringer (1961 1962) were followed except that the assay mediums of glyceraldehyde 3 phosphate dehydrogenase and phosphoglycerate kinase also contained 4 μmoles of 2 3 diphosphoglycerate to depress the diphosphoglyceromutase (E C 2 7 5 4) reaction

Adenylate kinase activity was estimated by coupling ADP formation with pyruvate kinase phosphoenolpyruvate lactic acid dehydrogenase and NADH Correction was made for the slow reaction obtained in the absence of AMP

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coefficient of $6\,22 \times 10^3/cm^2$ for both NADH and NADPH was assumed (Horecker and Kornberg 1948) Enzyme activities are expressed in units One unit is defined as that amount of enzyme which converts one μmole of pyridine nucleotide per min at 25° C

Intact red cells to be analyzed for enzyme activities were diluted with 0 15 *M* sodium chloride or 0 15 *M* sodium chloride containing 6 % Dextran giving a red cell suspension with a hematocrit of 10 % 50—150 μliters of this suspension were transferred to a medium containing the buffered assay solution and an amount of sodium chloride giving a final osmotic pressure of the medium of 305—310 milliosmols In the experiments accounted for in Table 4 the amount of sodium chloride added was increased to give an osmotic pressure of the assay medium of 395—400 milliosmols The final volume of the assay mixture was 3 0 ml

Accurate readings were difficult to achieve with the red cells in the light path The readings were therefo[illegible]

Acta physiol scand 1969, 76, 312—320

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Thus the present investigation deals with the determination of glyceraldehyde 3-phosphate dehydrogenase phosphoglycerate kinase and adenylate kinase (ATP AMP phosphotransferase E C 2 7 4 3) at the red cell surface, as well as that in a total hemolysate Various other enzymes of the red cell were likewise tested for activity at the surface and in the hemolysate

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TABLE I Enzyme activities of intact red cells
All enzyme activities are given in units per ml of red cells Numerals in brackets denote results obtained with 6 % Dextran T 40 in the assay medium For definition of a unit and for further details see Methods

	Supernatant		Intact red cells (338 μmoles of NaCl in assay medium)		Hemolysate (338 μmoles of NaCl in assay medium)		Hemolysate	
Glyceraldehyde 3 phosphate dehydrogenase (n=14)								
$\bar{X}$	0	(0)	0.34	(0.35)	11.1	(11.3)	16.5	(16.7)
S D			0.0236	(0.0225)	0.79	(0.69)	1.00	(0.95)
Phosphoglycerate kinase (n=14)								
$\bar{X}$	0.01	(0.01)	0.78	(0.79)	17.5	(17.5)	17.3	(17.2)
S D			0.0479	(0.0424)	1.21	(1.23)	1.13	(1.16)
Adenylate kinase (n=12)								
$\bar{X}$	0.07	(0.08)	0.28	(0.31)	14.1	(14.1)	14.8	(14.8)
S D			0.0446	(0.0383)	0.72	(0.75)	0.60	(0.52)

The isotonic hemolysate incubated once with red cells was mixed with a new portion of intact cells and the above procedure was repeated Finally the isotonic hemolysate incubated twice was mixed with a third new portion of red cells and the procedure with centrifugations and enzyme determinations was repeated once more

Statistical methods[1]

In the tables the different groups are described by expressing each attribute in terms of means ($\bar{X}$) and standard deviations (S D) The calculations were performed according to Snedecor (1956)

Results

Enzyme activities of intact red cells

The assay medium was made isotonic to maintain the integrity of the red cells during measurement of enzyme activity To achieve isotonic conditions, 338 μmoles of sodium chloride were added to the assay medium giving an augmentation of the osmotic strength of about 208 milliosmols The final osmotic pressure of the assay medium was thus 305—310 milliosmols

The results given in Table I clearly show that glyceraldehyde-3 phosphate dehydrogenase and phosphoglycerate kinase were detectable only if intact red cells were present in the assay medium The supernatant obtained after centrifugation of a prewarmed red cell suspension did not show any measurable activity The adenylate

[1] The statistical calculations were made by Dr S Nilsson Statistical Department Agricultural College of Sweden

TABLE IV Enzyme activity at a higher osmotic strength
Enzyme activities are given in units per ml of red cells For definition of a unit and for further details see text

	310 milliosmols in assay medium	400 milliosmols in assay medium
Red cells		
glyceraldehyde-3 phosphate dehydrogenase	0 36	0 24
phosphoglycerate kinase	0 80	0 81
adenylate kinase	0 28	0 27
Hemolysate		
glyceraldehyde-3 phosphate dehydrogenase	11 2	10 8
phosphoglycerate kinase	17 8	17 6
adenylate kinase	13 9	14 0

which have been suggested to be related to the structural integrity of the cell surface (Benesch and Benesch 1954)

No pyruvate kinase could be identified at the cell surface Thus the formation of extracellular ATP (Ronquist 1968) could not be accounted for to any appreciable extent by this enzyme

Effect of incubating red cells with hemolysate

A possible explanation of the presence of glyceraldehyde 3 phosphate dehydrogenase phosphoglycerate kinase and adenylate kinase at the surface of the cells is that the enzymes e g after small changes in shape of the cells would pass through the membrane to the outside There the enzymes would be attached to the surface structure by some kind of binding forces

Washed red cells were therefore analyzed for surface enzymes before and after incubation in 2 5 volumes of an isotonic hemolysate It is evident from the data in Table III that there was neither an augmentation of the surface enzymes nor a definite loss of the enzymes of the hemolysate used in the incubation experiment

Enzyme activity at a higher osmotic strength

Glyceraldehyde 3 phosphate dehydrogenase phosphoglycerate kinase and adenylate kinase were also tested in a medium containing 446 μmoles of sodium chloride i e a total osmotic pressure of the medium of 395—400 milliosmols Hypertonic hemolysis is not to be expected in such a medium (cf Zade Oppen 1968)

The figures in Table IV show that phosphoglycerate kinase and adenylate kinase were not affected by the higher osmotic strength The glyceraldehyde 3 phosphate dehydrogenase on the other hand was reduced and the reduction seemed to be more pronounced for the surface bound enzyme than for the intracellular glyceraldehyde 3 phosphate dehydrogenase This discrepancy could be explained by different

physico-chemical properties of the soluble and the surface bound enzyme However, another explanation might be that the red cells, by contraction in the hypertonic medium, made the surface-bound glyceraldehyde-3 phosphate dehydrogenase less available to its substrates and cofactors in the extracellular space

Discussion

The activity of glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase and adenylate kinase at the surface of the red cells was 3 1 %, 4.5 % and 2 0 %, respectively, of the total The degree of hemolysis during the assay procedure did not exceed 1 % The presence of 6 % Dextran T 40 in the assay medium did not reduce the surface enzyme activities to any extent. Consequently, the present results give strong vidence for the view that part of the glyceraldehyde-3 phosphate dehydrogenase, phosphoglycerate kinase and adenylate kinase of the red cells is bound to the outer surface

The supernatants (sodium chloride or sodium chloride Dextran centrifugate from the red cell suspensions) contained insignificant glyceraldehyde 3 phosphate dehydrogenase and phosphoglycerate kinase activity, indicating that no significant leakage of intracellular enzymes into the medium had occurred Some of the adenylate kinase activity, on the other hand, seemed to be easily eluted by the sodium chloride and sodium chloride Dextran solution This finding agrees with previous studies on red cells (Ronquist 1968), tumor cells (Ronquist and Ågren 1968) and on muscle cells (Dunkley, Manery and Dryden 1966 Manery, Riordan and Boegman 1967)

The cell surface bound activity of phosphoglycerate kinase as measured, was 2 3 times that of glyceraldehyde-3 phosphate dehydrogenase (Table I) On the other hand an inverse ratio between the activity of the two enzymes was obtained with an isolated membrane fraction from human erythrocytes (Ronquist 1967) Thus, the glyceraldehyde 3 phosphate dehydrogenase activity can be considered to be more firmly bound to the membraneous structure

I wish to express my gratitude to Professor Gunnar Agren for his valuable advice and encouragement during the course of the work

My thanks are also due to Mr Tage Fransson for skilful technical assistance

The work was supported by grants to Professor G Agren from the Swedish Medical Research Council (project No B 68-13X 228-04C) and by grants from the Medical Faculty University of Uppsala, and Svenska Sallskapet for Medicinsk Forskning

References

ÅGREN G and G RONQUIST Formation of extracellular adenosine triphosphate by tumour cells *Acta physiol scand* 1969 *75* 124—128

BENESCH R E and R BENESCH Relation between erythrocyte integrity and sulfhydryl groups *Arch Biochem* 1954 *48* 38—42

BOEHRINGER C F and SOEHNE GmbH Mannheim Germany Information January 1961, November 1961 and January 1962

CROSBY W H and D N HOUCHIN Preparing standard solutions of cyanmethemoglobin *Blood* 1957 *12* 1132—1136

DIMANT E E LANDSBERG and I M LONDON The metabolic behavior of reduced glutathione in human and avian erythrocytes *J biol Chem* 1955 *213* 769—776

DUNKLEY C R., J F MANERY and E E DRYDEN The conversion of ATP to IMP by muscle surface enzymes *J cell comp Physiol* 1966 *68* 241—247

HARBOE M A method for determination of hemoglobin in plasma by near ultraviolet spectrophotometry *Scand J clin Lab Invest* 1959 *11* 66—70

HARLEY J D Role of reduced glutathione in human erythrocytes *Nature* (Lond) 1965 *206* 1054—1055

HJELM M S G ÖSTLING and A E G PERSSON The loss of certain cellular components from human erythrocytes during hypotonic hemolysis in the presence of Dextran *Acta physiol scand* 1966 *67* 43—49

HORECKER B L and A KORNBERG The extinction coefficients of the reduced band of pyridine nucleotides *J biol Chem* 1948 *175* 385—390

MANERY J F J R RIORDAN and R J BOEGMAN Enzyme activity at muscle surfaces *Fed Proc* 1966 *26* 591

RONQUIST G Formation of adenosine triphosphate by a membrane fraction from human erythrocytes *Acta chem scand* 1967 *21* 1484—1494

RONQUIST G Formation of extracellular adenosine triphosphate by human erythrocytes *Acta physiol scand* 1968 *74* 594—605

SNEDECOR G W *Statistical methods applied to experiments in agriculture and biology* The Iowa State College Press Ames Iowa 5th ed 1956

VOGELL W F R BISHAI TH BÜCHER M KLINGENBERG D PETTE and E ZEBE Über strukturelle und enzymatische Muster in Muskeln von Locusta migratoria *Biochem Z* 1959 *332* 81—117

WARREN J C and S G CHEATUM Effect of neutral salts on enzyme activity and structure *Biochemistry* 1966 *5* 1702—1707

WARREN J C L STOWRING and M MORALES The effect of structure-disrupting ions on the activity of myosin and other enzymes *J biol Chem* 1966 *241* 309—316

ZADE OPPEN A M M Posthypertonic hemolysis in sodium chloride systems *Acta physiol scand* 1968 *73* 341—364

Acta physiol scand. 1969. 76. 321—334

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Single Unit Recordings from Muscle Nerves in Human Subjects

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Received 18 September 1968

Abstract

HAGBARTH, K.-E. and Å. B. VALLBO. *Single unit recordings from muscle nerves in human subjects.* Acta physiol. scand. 1969. 76. 321—334.

Single unit impulses were recorded with semi-microelectrodes from muscular portions of peripheral limb nerves in awake human subjects. Thirteen units were studied. Four of them could not be activated by mechanical peripheral stimuli but they fired steadily during sustained voluntary contractions. They were considered to be either motor fibres or proprio-[illegible]

[illegible] muscle nerves indicate that the muscle spindle afferent discharge is increased during voluntary contractions and thus, that the fusimotor system is engaged in voluntary motor acts.

As previously shown (Hagbarth and Vallbo 1967 b, Vallbo and Hagbarth 1968) nerve signals can be recorded with tungsten semi-microelectrodes percutaneously inserted into peripheral nerves of awake human subjects. Responses to adequate stimuli of mechanoreceptors in glabrous and non-glabrous skin have been analysed in a series of multi-fibre recordings and single unit recordings from cutaneous nerves (Vallbo and Hagbarth 1968) and a description has also been given of multi-fibre discharge patterns occurring in [illegible]uman muscle nerves during voluntary contraction, muscle stretch and other man[illegible]euvres liable to affect muscular mechanoreceptors (Hagbarth and Vallbo 1967[illegible], Vallbo 1967, Hagbarth and Vallbo 1968). The present report dealing with [illegible] series of single unit recordings from muscle nerves in healthy subjects provid[illegible] additional data concerning the discharge characteristics of human muscle re[illegible]ptors. A relatively large proportion of the units encountered had such dischar[illegible] characteristics as might be expected of muscle spindle afferents which are toni[illegible]lly activated by fusimotor outflow during voluntary contractions.

Methods

A of the experimental arrangements have been given in a previous report (Hagbarth and Vallbo 1968). Afferent nerve impulses of muscular origin were searched with a tungsten electrode inserted into the tibial, peroneal or median nerves of two healthy adults (the authors). Several hundred insertions were made during thirty three experimental sessions. All of the criteria for nerve activity from deep structures have been discussed in a previous report (Hagbarth and Vallbo 1968). In short, the following clues were considered to be of particular importance: (a) dull deep paresthesias and muscle fasciculations induced by electrical stimulation, (b) absence of neural response to light skin stimuli but response to mechanical stimuli reaching deep structures, and (c) discharge during voluntary contractions of the muscles in which the paresthesias were felt.

Various tests were performed in order to define the discharge characteristics of the single units. The test program was similar to the one used in a previous report (Hagbarth and Vallbo 1968): (a) local mechanical stimuli such as taps, slowly rising and sustained pressure, (b) passive joint movements, (c) voluntary contractions—either with the joint being fixed ("isometric") or free to move ("isotonic"). During these tests, great precautions were taken in order to avoid manoeuvres which could venture the recording situation. Therefore all voluntary muscle contractions were weak: a rough estimate indicated that their relative strength did not exceed 10 % of the maximum strength possible. Muscle vibrations and fast muscle twitches were totally avoided. The recording situation was particularly sensitive to movements of the ankle joint when the electrode was in the tibial nerve, in the popliteal fossa. The range of movement was thus small (20—30°) in these experiments. In spite of these precautions, the recording situation often deteriorated before the full test program had been completed and consequently the information concerning some of the units is fragmentary.

Results

Criteria for single unit recordings. In recordings from skin nerves (Vallbo and Hagbarth 1968) as well as from muscle nerves (Hagbarth and Vallbo 1968) the neural activity mostly appeared as multi-fibre discharges: a dense succession of deflections of various amplitudes, with the most conspicuous phase in the negative direction. However, as found in the skin nerve study, discharges from single nerve fibres could sometimes be discriminated with the present recording technique, and some of the muscle nerve recordings also comprised repetitive spikes of uniform size and shape indicative of single unit activity. Examples are shown in Fig. 1—3. The spikes had often larger amplitudes than the multi-fibre discharges and they had a large positive phase. Because of their size and shape, such impulses often stood out clearly from a background of noise and multi fibre discharge.

It may be argued that the impulses shown in the figures are not uniform enough to justify the conclusion that they were single unit discharges, as the spike amplitudes often varied by a factor of two from one impulse to the other. However, since the amplitude ratio of spikes to base-line was not more than between two and four, it seems very likely that interference from the background activity and noise could explain this variation. Furthermore, the variation looks more pronounced when the time scale is comparatively compressed as in the figures shown. When the tape recordings were displayed on an extended time scale, successive cycles of low frequency noise were separated from each other and it could then be seen that the spike amplitudes were more uniform. There was also a long term reduction of the spike size during the time of the experiment. An example is shown in Fig. 1 C. The recording on the right of the figure was taken earlier during the experiment than the two recordings on the left in which the impulses were also considerably smaller. Therefore, it seems that the variation of spike amplitudes, as seen in most of the figures, does not seriously invalidate against the conclusion that activities from single units were recorded. However, it is possible that Fig. 4 shows impulses from several units, but it seems justified to regard at least the large diphasic deflections as successive impulses from the same unit.

TABLE I Basic characteristics of afferent units in schematic form See text for further details Columns 5 and 6 refer to discharges during contractions of the muscles given in column 3 The dynamic sensitivity was estimated qualitatively, high' indicates that the discharge was clearly more pronounced during a stimulus increase than during a sustained stimulus

Unit no	Nerve recorded from	Probable site of end organ	Adaptation and subgroup	Discharge during "isotonic" contraction	Discharge during "isometric" contraction	Dynamic sensitivity	
1	median	flexor digitorum muscles	fast	increased	increased		Fig 1
2	tibial	gastrocnemius muscle	fast	increased	increased		
3	peroneal	peroneal muscles or tendons?	slow type A	decreased	no change	low	Fig 2
4	median	flexor digitorum muscles or tendons?	slow type A	decreased	no change	?	
5	peroneal	anterior tibial muscle	slow type B	not tested	increased	high	
6	peroneal	toe extensor muscles	slow type B	increased	increased	high	
7	tibial	gastrocnemius muscle	slow type B	not tested	increased	high	Fig 3
8	tibial	gastrocnemius muscle	slow type B	increased	increased	high	Fig 4
9	tibial	gastrocnemius muscle	slow type B	increased	increased	low	

Classification of the units The units described in the present investigation were recorded from the tibial the peroneal or the median nerves The electrode tip was placed in muscular portions of these nerves as determined by the criteria previously described (Hagbarth and Vallbo 1968) The end organs were all located in muscles or other deep structures of the leg or forearm Units with end organs in the hand or the foot were not included The same limitation was used in the previous study dealing with multi-fibre discharges from deep structures In all, 13 single units which fulfilled these requirements and which could easily be discriminated from the background activity were encountered Nine of the 13 units could be activated by local taps and/or sustained pressures which stimuli were effective only when applied to a restricted area over the muscle bellies or to particular muscle tendons The neural responses were closely related to the intensities and the time courses of the local stimuli whereas after-discharges were never seen, nor variations of the

response to successive identical stimuli. Thus it seems justified to conclude that these units were afferents. The basic findings concerning these units are given in Table I. The conclusion that the units were proprioceptors was based upon two main findings: (a) threshold stimuli were of such intensities that deformation of deep structures without doubt occurred; (b) their discharge in response to voluntary muscle contractions was more pronounced than the maximum discharge which could be induced by local mechanical stimuli. *However, it should be emphasized that a skin unit can be mistaken for a proprioceptor if local mechanical stimuli are applied not in the area of minimum threshold but in the periphery of the cutaneous receptive field.* It is very unlikely that such mistakes could have been made in the present study since great care was always taken in order to define the area where the threshold was minimum for mechanical stimuli.

Four of the proprioceptor units were found in the tibial, three in the peroneal and two in the median nerves. For seven of the afferents the end organs could be distinctly localized by mechanical stimuli. The receptive fields to local deformation from the skin surface were mostly in an order of size varying from 10 to 30 cm². For two afferent units (units no. 3 and 4 in Table I) the site of the end organs could not accurately be determined by means of local stimuli but receptor locations were inferred on the basis of their responses to joint movements and pressures on the muscle tendons. Two of the afferent units were fast adapting and seven of them were slowly adapting. The slowly adapting units were divided into two groups designated as type A and type B which will subsequently be described separately. *Four of the 13 units fired steadily during sustained voluntary contractions but they could not be activated by peripheral stimuli.*

Fast adapting units. Two fast adapting receptors were found, one in the calf region and the other one in the volar region of the forearm (units no. 1 and 2 in Table I). They were characterized by a high dynamic sensitivity and a fast adaptation to all kinds of sustained mechanical stimuli. The activity shown in Fig. 1 was recorded from the median nerve. The ending was located approximately 10 cm distally to the elbow as it is indicated by the black dot. It could be ascertained in several ways that this was not a skin unit: the median nerve does not innervate this skin area, and furthermore it could be shown during the experiment by pulling the skin sideways that the exact point at which a stimulus was effective was bound to a point in the deep structures *and not to the skin.* As it was determined by local mechanical stimuli, the receptive field of this particular unit was unusually small, that is approximately 1 cm². Responses to a local sustained pressure and taps are shown in Fig. 1 A. On the left of the figure it can be seen that two impulses were initiated at the beginning of a lasting stimulus of 2—3 newtons but that there was no sustained discharge. The responses to taps are depicted on a more extended time scale in the right hand part of Fig. 1 A. The first two taps which reached a peak of approximately 2.5 newtons evoked two impulses while the last tap which had a more slowly rising phase induced only one single impulse. Consequently the receptor was not particularly sensitive to local deformations from the skin surface but

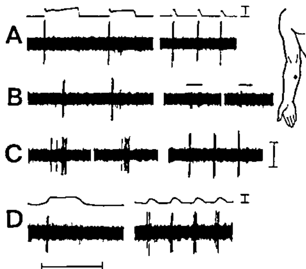


Fig 1 Response to mechanical stimuli and voluntary contractions of a fast adapting unit, recorded from the median nerve The location of the receptor is indicated by a black dot on the drawing A Local sustained pressures and taps The stimulus force and time course are indicated by the upper trace Note that the time scale is different for the right hand and the left hand parts (see below) B left Fast passive extensions of the metacarpo-phalangeal joints B right Fast passive flexions of the same joints Movements are indicated diagrammatically by bars above the nerve record C, left Fast active 'isotonic' extensions of the same joints C, right Fast active 'isotonic" flexions of the same joints D Isometric contractions of the finger flexors The upper trace indicates active forces exerted by the finger tips In this and in all the other figures upward deflections in the neurograms indicate positive signals at the intraneural electrode

Calibrations Nerve signal amplitude bar opposite C 20 μV Stimulus force bar opposite A 2 5 newtons Active force bar opposite D 5 0 newtons Time 2 5 sec for A left 1 0 sec for all other traces

it was readily excited by muscle movements as shown in Fig 1 B, C, and D Short bursts of impulses appeared when the three ulnar fingers were passively extended by a fast blow on the finger tips (Fig 1 B left), but there was no response to fast passive flexions (Fig 1 B, right) Voluntary contractions, resulting in fast active movements of the same fingers, were more effective stimuli than passive movements Fast extensions induced five to ten impulses with a maximum frequency of approximately 70 imp/sec as it was calculated after five successive spike intervals (Fig 1 C, left) Active flexions also excited the ending in contrast to passive flexions (Fig 1 C, right) Furthermore, "isometric' contractions of the finger flexor muscles elicited a discharge simultaneously with the rise of the muscle force, as it was recorded with a strain gauge device upon contact with the finger tips (Fig 1 D) Therefore large changes of muscle length were not essential This receptor apparently did not specifically signal the rate of change of the muscle length or of the tension, but responded to various types of rapid muscle movements It seems that, for example a Pacinian corpuscle, located in the muscle fascia, might show this type of discharge (Hunt and McIntyre 1960, Hunt 1961, Barker 1962) The other fast adapting unit which was located laterally in the proximal calf region had very similar discharge characteristics as the one described

Slowly adapting type A units The two receptors in this group (units no 3 and 4 in Table I) could not be directly located by mechanical stimuli applied to a re-

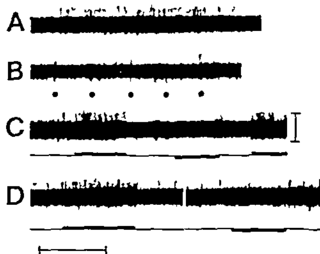


Fig 2 Response to mechanical stimuli and joint movements of a slowly adapting unit recorded from the peroneal nerve A Sustained pressure on the peroneal tendons 5 0 cm proximal to the lateral malleolus. B Taps on the peroneal tendons The impulses induced by taps are indicated by black dots below the trace of the nerve activity Note the difference in time scale between B and the other traces (see below) C Passive joint movements which are diagrammatically indicated below the trace of the nerve activity Bars above the line indicate a supination of the foot (muscle stretch), and the bar below the line indicates a pronation of the foot (muscle shortening) D Active "isotonic" joint movements indicated as in C. Movements were of approximately the same range in C and D

Calibrations Nerve signal amplitude 20 µV Time 5 0 sec for A C and D 1 0 sec for B

stricted area over the muscle bellies Their locations were inferred from their responses to joint movements and pressures on muscle tendons One was located in or close to the peroneal muscles or tendons, and the other, in the volar region of the forearm probably in close association with the finger flexor muscles They were activated by passive as well as active joint movements which caused an extension of the structures in which they were located Contrary to all other units they were not activated by weak isometric muscle contractions The discharge patterns of the peroneal unit are shown in Fig 2 It responded readily to pressure on the peroneal muscle tendons The discharge associated with a lasting pressure on these tendons at a point five cm proximal to the lateral malleolus is depicted in Fig 2A This activity showed very little adaptation and it was possible to keep the unit firing to such stimuli for several minutes Responses to tendon taps are shown in Fig 2B The time scale is expanded by a factor of five, compared to the other records in this figure During this test the foot was passively held in supination in order to make the muscle tendons more accessible This extension of peroneal muscles was associated with a sustained discharge (see below) The impulses induced by tendon taps are marked by black dots below the trace Only one positive spike appeared in response to each tap which suggested that this unit had a poor dynamic sensitivity

Passive joint movements altered the discharge markedly In the test illustrated in Fig 2C, the unit fired with a frequency of approximately 5 0 imp/sec when the foot was kept in an intermediate position. A passive supination involving a stretching of the peroneal muscles resulted in an increase of the impulse frequency to approximately 15 imp/sec When an intermediate position was reached again there was no discharge at all in this particular test The position of the foot might have been slightly different before and after supination and this could be the main reason for

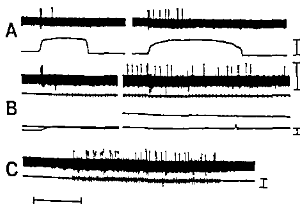


Fig 3 Response to local mechanical stimuli joint movements and muscle contractions of a slowly adapting unit recorded from the tibial nerve The EMG was recorded with surface electrodes over the medial gastrocnemius muscle A Local mechanical stimuli The muscle was relaxed the ankle joint was at an angle of 90° The stimulus force and the time course are indicated by lower traces B Discharge induced by (a) a passive dorsal flexion of the foot (lowermost trace) (b) a local sustained pressure (second trace from below) and (c) a weak muscle contraction The run started with a passive dorsal flexion of the foot and a slight voluntary activation In this situation a sustained local pressure was exerted on the same spot as in A the first few seconds were cut out at the gap on account of artefacts C Discharge during an isometric voluntary contraction (The triangular shaped deflections in traces of the force and the angle in A and B are artefacts due to minute variations of the tape speed)
Calibrations Nerve signal amplitude bar opposite B 20 μV EMG signal amplitude bar opposite C 200 μV Local mechanical stimuli bar opposite A 5 0 newtons Ankle joint angle small bar opposite B indicates 90° (bottom) and 84° (top) Time 5 0 sec

the difference in the single unit discharge When the foot was passively moved in the other direction and kept in pronation implying a passive shortening of the muscles the unit remained silent However on the movement back to intermediate position a short burst of impulses was elicited Another passive supination induced a discharge of similar frequency as the first one

Responses to active movements are illustrated in Fig 2 D The impulse frequency changed approximately from 7 0 to 14 imp/sec upon active supination of the foot In the right hand part of Fig 2 D the discharge frequency was approximately 9 0 imp/sec when the foot was kept in an intermediate position A voluntary activation of the peroneal muscles resulting in a pronation of the foot and a shortening of these muscles stopped the discharge completely By contrast isometric contractions of the peroneal muscles or their antagonists did not affect the discharge of the unit The two units of the A type were thus characterized by the fact that their discharge changed with joint position (or muscle length) in the same way whether the movements were active or passsive The exact site and nature of the end organs remains obscure

Slowly adapting type B units These units (no 5—9 in Table I) responded with an increased discharge to passive muscle stretch like the two units described in the earlier section However unlike the type A units they were all activated by weak isometric voluntary contractions and an increased discharge could be demonstrated for three of them during isotonic contractions of the muscles in which the receptors were located All the units in the B group could be activated by local

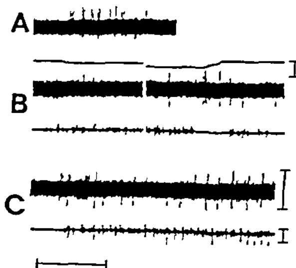


Fig. 4. Response to a local pressure, "isotonic" and "isometric" voluntary contractions of a slowly adapting unit recorded from the tibial nerve. The end organ was probably located in the gastrocnemius muscle. A. Local sustained pressure. Stimulus not indicated. B. "Isotonic" voluntary contraction. The ankle joint movement is indicated by an analogue signal above the trace of the nerve discharge. The EMG activity is indicated below. C. "Isometric" voluntary contraction. *EMG activity below as in B.*

Calibrations: Nerve signal amplitude: bar opposite C, 20 μV. EMG signal amplitude: bar opposite C, 100 μV. Ankle joint angle: bar opposite B indicates 110° (bottom) and 90° (top). Time: 1.0 sec.

mechanical stimuli within a limited area over the muscle bellies. Three of them were located in the ankle extensor muscles, probably gastrocnemius, and the other two in the ankle flexors.

The activities of two slowly adapting type B units are illustrated in Fig. 3 and 4. The discharge shown in Fig. 3 was recorded from the tibial nerve. The receptor could be excited by a strong local pressure applied to the proximal part of the calf region. Judging from the response to such stimuli, the ending seemed to be located quite deeply, probably in the medial head of the gastrocnemius muscle. Figure 3 A *shows the nerve discharge upon local pressure during relaxation. It can be seen that* a few impulses were induced at the beginning of the stimulus, but that there was no lasting discharge. Likewise, a sustained passive dorsiflexion of the foot elicited a few impulses while the foot was moving, but no sustained discharge (Fig. 3 B, left). The subject unintentionally made a weak contraction of the ankle extensor muscles while his foot was held in dorsiflexion, as it can be seen in the EMG record. While this contraction persisted in the stretched muscles, a sustained local pressure (similar to the one shown in Fig. 3 A) was again applied in the upper calf region, and in this situation a continuous discharge was induced (Fig. 3 B, right). Such an effect *could not be attained by the local pressure or the passive muscle stretch alone. How*ever, stronger "isometric" voluntary contractions of the calf muscles, were sufficient by themselves to make the unit fire continuously (Fig. 3 C). It can be seen that the discharge was irregular and that the frequency was low, approximately 2.0 and 3.5 imp/sec in Fig. 3 B and C, respectively. Four of the units in this group (no. 5–8 in Table I) exhibited similar discharge characteristics as the unit illustrated in Fig. 3, that is to say they responded with only a shortlasting discharge to a sustained passive muscle stretch or a local pressure, whereas they gave a continuous discharge when the muscle was engaged in a voluntary contraction. During voluntary activations irregular discharges were seen from three of the type B units.

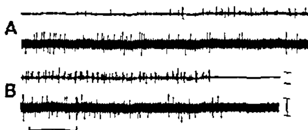


Fig 5 EMG and single nerve unit discharge during an isometric voluntary contraction Upper trace EMG recorded with surface electrodes over the anterior tibial muscle Lower trace Nerve impulses recorded from the peroneal nerve at the level of the knee joint The unit was possibly efferent A and B are continuous
Calibrations EMG signal amplitude 100 µV Nerve signal amplitude 20 µV Time 1 0 sec

All the units in this group were tested for their response to isometric voluntary contractions, three of them were also tested for their response to isotonic contractions an example of which is shown in Fig 4 These muscle activations were all associated with sustained discharges Whether one type of contraction was more effective than the other, it could not be unequivocally shown but the findings suggest that 'isometric contractions were associated with a slightly higher discharge frequency, the EMG activity being of a similar intensity

The time relation between the onset of the EMG activity and of the proprioceptor discharge was carefully observed during voluntary contractions The EMG activity usually started before or simultaneously with the discharge of the afferent unit but some exceptions to this rule were seen (Fig 4 C) However it was hard to exclude that low threshold motor unit potentials had escaped detection in such cases

One unit (no 9 in Table I) located in the gastrocnemius muscle differed from the other units in this group in that it had a poor dynamic sensitivity and that it fired spontaneously when the muscles were relaxed and the foot was in an intermediate position The mean frequency was approximately 6 0 imp/sec None of the other units showed a similar spontaneous discharge

It seems likely that most of these units were muscle spindle afferents The arguments for this conclusion will be given in the discussion.

The impulse frequencies induced by the various tests were low as compared to many findings in the cat and the monkey (*e g* Matthews 1933 Jansen and Matthews 1962 Critchlow and von Euler 1964 Crowe and Matthews 1964 a Koeze Phillips and Sheridan 1968) The maximum sustained frequency seen in the present study was approximately 30 imp/sec. This was recorded during an isometric contraction from a unit probably located in the anterior tibial muscle (unit no 5 in Table I)

Efferent units (?) Four units did not fulfill the main criterion of afferent units in that they could not be activated by peripheral mechanical stimuli They were all recorded from the peroneal nerve and they were characterized by a sustained discharge during contractions of the muscles innervated by the peroneal nerve As

an example single unit impulses and the EMG activity, which was recorded simultaneously with surface electrodes overlying the anterior tibial muscle are shown in Fig 5 In the sequence illustrated the subject made an isometric contraction of a moderate intensity of the ankle flexor muscles It can be seen that the nerve unit discharged with regular spike intervals, except for the first few seconds The maximum frequency was approximately 25 imp/sec The other three units in this group also exhibited a regular discharge but the maximum frequencies were somewhat lower (10—20 imp/sec) during isometric voluntary contractions of moderate strength It seems very likely that most of the units in this group were *a* motor fibres but it cannot be excluded that at least some of them were proprioceptor units which during relaxation had a high threshold to peripheral stimuli

Discussion

Two obstacles encountered in most single unit recordings were particularly hard to overcome in experiments on awake human subjects One concerns the stability of the recording situation In our experience a floating electrode inserted by hand has definite advantages as compared to a more rigid external fixation of the electrode Still the present recording situation was too unstable to permit such tests as muscle twitches muscle vibrations or strong voluntary contractions The other obstacle arises from the fact that the preparation is intact There are *a priori* many possibilities to consider when trying to determine the origin of a unitary discharge a mixed nerve it may be efferent or afferent it may originate from a receptor and deep or superficial structures etc Furthermore in an intact preparation there is no easy way of stimulating deep structures without also affecting receptors in the skin *On the other hand an alert human subject can tell about the site* and the quality of the insertion paresthesias an information which is of great help when trying to identify the neural structures recorded from and he can make the active movements and isometric muscle contractions desired for the analyses of proprioceptor discharges

The single units were separated in four groups This grouping is largely descriptive since the available information does not allow an unequivocal classification in efferents *Golgi tendon organs muscle spindle afferents etc However it seems* meaningful to consider which types of receptor units might constitute the various groups as presented here Such a consideration must be based upon the assumption that humans are provided with proprioceptors of similar properties as other mammals

One group of units fired during voluntary contractions but no discharge could be induced by peripheral mechanical stimuli This suggests that they were efferents Since the recording technique most likely gives a selection of large diameter nerve fibres (Vallbo and Hagbarth 1968) the efferent units encountered probably were *a* motor fibres rather than fusimotor fibres The discharge characteristics of the units in this group largely agreed with such an interpretation (Bigland and Lippold 1954

Harrison and Mortensen 1962) It is possible that some of the units in this group were in fact afferents the receptors of which had high thresholds to mechanical stimuli from the outside but were readily activated by muscle contractions Such responses can be expected from some Golgi tendon organs (Jansen and Rudjord 1964 Houk and Henneman 1967)

Two afferent units were encountered showing short lasting discharge of comparatively high impulse frequency to all kinds of tests employed Such discharge characteristics suggest that the end organs were Pacinian corpuscles (Hunt and McIntyre 1960 Hunt 1961 Barker 1962)

The slowly adapting afferents were divided in two groups The type A units were not activated during weak isometric voluntary contractions but they responded to joint movements Their discharge increased to movements in one direction and decreased to movements in the other direction independently of whether these joint movements were active or passive The end organs could not be exactly located by peripheral stimuli but some findings suggest that they were situated in the muscles or the tendons Their discharge increased when these structures were stretched The receptors were probably not Golgi tendon organs since they did not respond to an active rise of tension during isometric contractions but they responded to passive muscle stretch which did not give rise to an appreciable muscle tension They could be spindle afferents which were unloaded during voluntary contractions or since it could not be definitely excluded that they were located in the joint capsule or ligaments they could be joint receptors (Boyd and Roberts 1953 Skoglund 1956)

The type B slowly adapting afferents fired during weak isometric voluntary contractions they responded to passive muscle extension and some of them to active muscle shortening Since they were all located in the fleshy parts of the muscle it was concluded that they were either spindle afferents or Golgi tendon organs So far it has not been possible in the single unit recordings to employ the common test discriminating between tendon organs and spindle afferents *i e* to determine the receptor response to a muscle twitch evoked by an electrical stimulation of the nerve trunk nor has any other test provided conclusive evidence for a differentiation in this respect However some findings suggest that the majority of these units were spindle primary endings rather than secondary endings or tendon organs On the one hand it is unlikely that any large proportion of these units was group II spindle afferents as the recording technique probably gives a selection of large diameter fibres Furthermore with one striking exception the units had a comparatively pronounced dynamic sensitivity On the other hand several findings speak in favour of spindle afferents rather than Golgi tendon organs one finding being again the high dynamic sensitivity (Matthews 1933 Jansen and Rudjord 1964 Alnaes 1967 Houk and Henneman 1967) In general primary spindle endings have lower threshold than Golgi tendon organs to passive muscle stretch (Matthews 1933) although very high sensitivity has recently been demonstrated for some Golgi tendon organs in the cat Alnaes 1967) The weak test stimuli and the sm movements employed in the present study implies a bias towards low

units. Furthermore, irregular discharges, which were found for several of the type B units, have been described as a characteristic of spindle afferents subject to fusimotor drive (Eldred, Granit and Merton 1953, Crowe and Matthews 1964 b, Koeze, Phillips and Sheridan 1968). Therefore, it seems justified to conclude that a large portion of the slowly adapting type B units were in fact spindle primary afferents, although conclusive evidence could not be presented for any particular unit.

In a previous analysis of the multi-fibre discharge patterns in human muscle nerves, twitch tests indicated that a major part of the neural activity which was recorded during passive muscle stretch and active contractions, originated from muscle spindles (Hagbarth and Vallbo 1968). It was also shown that phasic responses to mechanical stimuli were pronounced, whereas a substantial lasting discharge was not readily induced, except by muscle vibration or "isometric" voluntary contractions. This agrees well with the present findings, since a relatively large proportion of the slowly adapting units (4 out of 7) responded readily to phasic local stimuli and sustained voluntary contractions, but not to a sustained local pressure, nor to a sustained passive muscle stretch. The two studies are complementary to each other in supporting the notion, that this type of response originated from group I spindle afferents.

The majority of the slowly adapting afferents fired during weak voluntary contractions of the muscles in which they were located, indicating that, simultaneously with the skeleto-motor activation, there was a contraction of the intrafusal muscle fibres. This provides a more direct evidence for the conclusion which was presented on the basis of the multi-fibre recordings, that the fusimotor system, besides being engaged in reflexes, postural mechanisms, and breathing, as shown in other mammals (e.g. Hunt 1951, Eldred, Granit and Merton 1953, Eldred and Hagbarth 1954, Critchlow and von Euler 1963, Granit 1968), is also activated during voluntary motor acts. This accords with investigations showing that spindle afferent discharges may be increased by an electrical stimulation of the motor cortex in the monkey (e.g. Koeze, Phillips and Sheridan 1968). The discharge frequencies of the afferents were mostly low. This might, at first sight, suggest that the system is not very powerful. On the other hand it should be recalled that the contractions were weak. Strong contractions might well give rise to much higher impulse frequencies. Anyhow, the findings do indicate that, during voluntary motor acts, the α motoneuron discharge is supported by afferent impulses from spindles, but the findings do not give any answer as to how pronounced this support may be in various situations. Some of the units, assumed to be spindle afferents, also fired during "isotonic" contractions, implying that these spindles were not unloaded by the active muscle shortening, but the decrease of muscle length was compensated by intrafusal contractions. No evidence was obtained in support of the idea that the motoneuron discharge was reflexively initiated by spindle afferents, as a consequence of fusimotor activations; e.g. the EMG activity usually started before or simultaneously with the discharge of the afferent units.

The majority of the slowly adapting receptors were located in the leg muscles and

their discharges were recorded when the subject was lying on a bench It should be stressed that spindle receptors in other muscles and in other situations might exhibit different discharge characteristics from those suggested by the findings reported in the present investigation

Two functionally different types of fusimotor fibres have been demonstrated in the cat although there is not complete agreement on the significance of the two systems (*e g* Matthews 1962, Crowe and Matthews 1964 a, Bessou and Laporte 1966, Schafer and Henatsch 1968) It seems premature to present any suggestions with regard to the two fusimotor systems in man A quantitative analysis of the muscle spindle responses to passive muscle stretch was not undertaken, but with some improvements of the present experimental technique, it may be possible to make such an analysis of the position and velocity sensitivity of human muscle spindles during various types of voluntary motor acts

This investigation was supported by the Swedish Medical Research Council (Project No B 67 14X 2075 01)

References

ALNAES E Static and dynamic properties of Golgi tendon organs in the anterior tibial and soleus muscle of the cat *Acta physiol scand* 1967 *70* 176—187

BARKER D The structure and distribution of muscle receptors In *Symposium on Muscle Receptors* Ed D Barker Hong Kong University Press Hong Kong 1962 227—240

BESSOU P and Y LAPORTE Observations on static fusimotor fibres In *Muscular afferents and motor control* Ed R. Granit Almqvist & Wiksell Uppsala 1966 81—89

BIGLAND B and O C J LIPPOLD Motor unit activity in the voluntary contraction of human muscle *J Physiol* (Lond) 1954 *125* 322—335

BOYD I A and T D M ROBERTS Proprioceptive discharges from stretch receptors in the knee joint of the cat *J Physiol* (Lond) 1953 *122* 38—58

CRITCHLOW V and C VON EULER Intercostal muscle spindle activity and its γ motor control *J Physiol* (Lond) 1963 *168* 820—847

CROWE A and P B C MATTHEWS The effects of stimulation of static and dynamic fusimotor fibres on the response to stretching of the primary endings of muscle spindles *J Physiol* (Lond) 1964 a. *174* 109—131

CROWE A and P B C MATTHEWS Further studies of static and dynamic fusimotor fibres *J Physiol* (Lond) 1964 b *174* 132—151

ELDRED E R GRANIT [illegible] significance *J Phys* [illegible]

ELDRED E and K E [illegible] of certain skin areas [illegible]

GRANIT R The func [illegible] *Med* 1968 *61* 69—78

HAGBARTH K E and A B VALLBO Afferent response to mechanical stimulation of muscle receptors in man *Acta Soc Med upsalien* 1967 a *72* 102—104

HAGBARTH K E and A B [illegible] semi microelectrodes in hur [illegible]

HAGBARTH K E and A B [illegible] ing muscle stretch and cor [illegible]

HARRISON V F and O A [illegible] unit activity in the tibialis [illegible]

HOUK J and E HENNEMAN [illegible] soleus muscle of the cat *J* [illegible]

HUNT C C The reflex act [illegible] *115* 456—469

HUNT C C On the nature of vibration receptors in the hind limb of the cat *J Physiol (Lond)* 1961 *155* 175—186
HUNT C C and A K MCINTYRE Characteristics of responses from receptors from the flexor longus digitorum muscle and the adjoining interosseus region of the cat *J Physiol (Lond)* 1960 *153* 74—87
JANSEN J K S and P B C MATTHEWS The central control of the dynamic response of muscle spindle receptors *J Physiol (Lond)* 1962 *161* 357—378
JANSEN J K S and T RUDJORD On the silent period and Golgi tendon organs of the soleus muscle of the cat *Acta physiol scand* 1964 *62* 364—379
KOEZE T H C G PHILLIPS and J D SHERIDAN, Thresholds of cortical activation of muscle spindles and α motoneurones of the baboon's hand *J Physiol (Lond)* 1968 *195* 419—449
MATTHEWS B H C, Nerve endings in mammalian muscle *J Physiol (Lond)* 1933 *78* 1—53
MATTHEWS P B C The differentiation of two types of fusimotor fibre by their effects on the dynamic response of muscle spindle primary endings *Quart J exp Physiol* 1962 *47* 324—333
SCHAFER S S and H D HENATSCH Dehnungsantworten der primaren Muskelspindel Afferenz bei elektrischer Reizung und naturlicher Innervation der beiden fusimotorischen Faser typen *Exp Brain Res* 1968 *4* 275—291
SKOGLUND S Anatomical and physiological studies of knee joint innervation in the cat *Acta physiol scand* 1956 *36* Suppl 124 1—101
VALLBO Å B Impulse patterns in a muscle nerve during voluntary contraction in man *Acta physiol scand* 1967 *69* 123—124
VALLBO Å B and K E HAGBARTH Activity from skin mechanoreceptors recorded percutaneously in awake human subjects *Exp Neurol* 1968 *21* 270—289

Acta physiol. scand. 1969. 76. 335—339
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The Mechanism of Histamine Release from Isolated Rat Peritoneal Mast Cells Induced by d-Tubocurarine

By

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Received 19 September 1968

Abstract

FRISK-HOLMBERG, M and B UVNÄS *The mechanism of histamine release from isolated rat peritoneal mast cells induced by d-tubocurarine* Acta physiol scand 1969 *76* 335—339

Isolated rat peritoneal mast cells were exposed to d tubocurarine and the release of histamine was studied under various conditions The proposal is made that d tubocurarine although a monomer induces histamine release by a mechanism similar to that one triggered by the polymer synthetic amine compound 48/80

Alam *et al* (1939) first showed that d tubocurarine releases histamine from skeletal muscle Since then several authors have reported histamine release from various tissues by this substance (Comroe 1946 Rocha-e Silva and Schild 1946, Mongar and Whelan 1953, Archer 1959 and Rothschild 1966, 1967)

It is believed that long chain monoamines such as octyl- and decylamine and other surface active agents degranulate mast cells and release their histamine by direct physical damage to the cell membrane On the other hand the degranulation and histamine release caused by certain polymeric compounds *e g* the synthetic polyamine compound 48/80 and various basic polypeptides seem to depend on the triggering of enzymatic processes in the mast cell membrane leading to an active discharge of granules from the cells (Thon and Uvnas 1967) The aim of the present investigation was to clarify the mechanism by which d tubocurarine, a monomer releases histamine from isolated rat peritoneal mast cells

Experimentel procedures

Isolation of mast cells

Male Sprague Dawley rats weighing 350—400 g were anaesthetized with ether and bled by cutting the carotids 7 ml of isotonic NaCl solution adjusted to pH 6 with 10 % Sorensen phosphate buffer was injected into the abdomen After gentle massage for 1 1/2 min the ab

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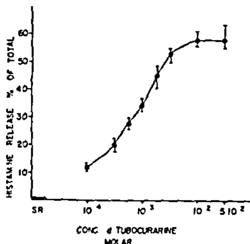


Fig 1

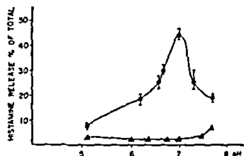


Fig 2

Fig 1 Release of histamine from intact mast cells by various concentrations of d-tubocurarine, 37° C SR = Spontaneous histamine release Each point represents the mean of three experiments, each carried out with duplicate samples

Fig 2 Effect of pH on histamine release from intact mast cells by d tubocurarine (2 mM) 37° C ●—●—● Spontaneous histamine release ▲—▲—▲ Each point represents the mean of three experiments carried out with duplicate samples (Vertical bars represents range in all figures)

and 40 % w/v as described by Uvnas and Thon (1961) After isolation and washing the cells were pooled and cell counts were performed in a Burker chamber after staining with toluidine blue

Incubation technique

Unless otherwise stated aliquots of the pooled cells were suspended in an isotonic solution (pH 7 containing 150 mM NaCl 2 7 mM KCl, 1 0 mM $CaCl_2$, 10 % Sorensen phosphate buffer and 0 1 % human serum albumin) and incubated with the releaser for 5 min at 37° C with constant shaking in a waterbath After the incubation the tubes were cooled in an icebath and then centrifuged (400 × *g*) for 10 min at 4° C Where appropriate an aliquot of the incubation mixture was inspected under the microscope to determine if degranulation had occurred

Determination of histamine

After boiling the supernatants and sediments for 5 min the histamine was determined by a slight modification of the fluorometric method of Shore Burkhalter and Cohn (1959) (The perchloric acid step was omitted as no interfering amino acids or proteins were present) The fluorescence was measured in a Farrand photoelectric fluorometer model A 3 In the concentrations used d tubocurarine does not interfere with the estimation of histamine by this method

Materials

Ficoll (mol wt 400 000 was obtained from AB Pharmacia Uppsala Sweden human serum albumin from AB Kabi Strangnas Sweden and d tubocurarine dihydrochloride from AB Kabi Strangnas Sweden and d tubocurarine dihydrochloride from AB Sigma Sweden Isotonic sucrose (0 28 0 31 M) in distilled water was adjusted to pH 7 with NaOH (10^{-4} M) Other substances were obtained from normal commercial sources

Results

Dose-response relationship

d-Tubocurarine caused degranulation and histamine release in concentrations above 10^{-4} M (Fig 1) A maximal release of about 50 % of the total histamine content

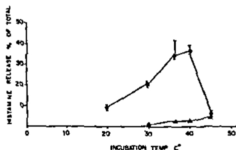


Fig 3

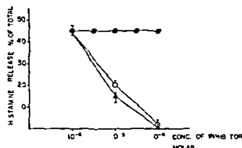


Fig 4

Fig 3 Effect of incubation temperature on histamine release from intact mast cells induced by d tubocurarine (2 mM) ●—●—● Spontaneous histamine release ▲—▲—▲ Each point represents the mean of three experiments carried out with duplicate samples

Fig 4 Inhibition of histamine release induced by d tubocurarine (2 mM) from intact mast cells by pretreatment with ninhydrin ○—○—○ or ethylmaleimide ▲—▲—▲ Release with d tubocurarine alone ●—●—● 37° C Spontaneous release deducted Each point represents the mean of three experiments carried out with duplicate samples

was obtained with a concentration of 5 10^{-3} M and could not be surpassed even with a tenfold increase in the d tubocurarine concentration

The release of histamine by d tubocurarine occurred over a fairly narrow pH range with an optimum between pH 6 6—7 4 (Fig 2)

Influence of temperature

The histamine release induced by d tubocurarine increased with temperature being optimal between 37° C—40° C (Fig 3) Above this temperature there was a rapid fall the release being totally abolished at 45° C This loss of responsiveness of the mast cells to d tubocurarine was irreversible

Influence of enzyme inhibitors

The releasing action of d tubocurarine was prevented by preincubation the cells with either ninhydrin (blocking amino groups) or N ethylmaleimide for 15 min before adding the liberator Inhibition of histamine release was seen with inhibitor concentrations above 10 molar (Fig 4) The sensitivity of inhibitor treated cells to the releasing action of d tubocurarine was not restored by washing the cells once in buffered saline before adding the liberator

2 4 Dinitrophenol DNP) an uncoupler of aerobic high-energy phosphate synthesis blocked histamine release in concentrations above 10^{-6} M The presence of glucose 1 mg ml almost completely abolished the inhibitory action of this concentration of DNP (Fig 5) In these experiments the cells were preincubated for 15 min with glucose The inhibitor was added and after further 15 min the liberator was added Thyroxine another uncoupler of oxidative phosphorylation blocked the amine liberation in concentrations above 10^{-6} M (Fig 6) This block was reversed by preincubation with glucose

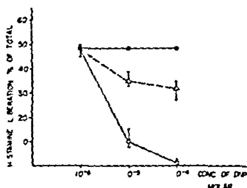


Fig 5

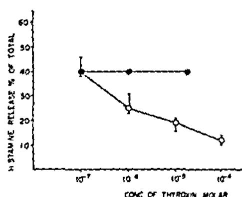


Fig 6

Fig 5 Inhibition of histamine release induced by d tubocurarine (2 mM) from intact mast cells by pretreatment with DNP ○—○—○ and reversal of this inhibition by preincubation with glucose (1 mg ml) △—△—△ Release with d tubocurarine alone ●—●—● 37° C. Spontaneous release deducted. Each point represents the mean of three experiments carried out with duplicate samples

Fig 6 Inhibition of histamine release induced by d tubocurarine (2 mM) from intact mast cells by pretreatment with thyroxine ○—○—○ Release with d tubocurarinealone ●—●—● 37 C Spontaneous release deducted Each point represents the mean of three experiments carried out with duplicate samples

Discussion

The present study indicates that d tubocurarine induced degranulation and histamine release by a mechanism similar to that triggered by compound *48/80* *The mast* cell response was temperature dependent *At 0° C there was no histamine release The release reached its maximum between 37—40° C* Above 40° C the mast cell response decreased and above 45° C the cells irreversibly lost their responsiveness to d tubocurarine The temperature dependence is consistent with the assumption *that enzymatic processes are involved in the mast cell reaction Further evidence for* enzymatic participation is the narrow pH range (6 8—7 2) over which release occurs and the blocking action of enzyme inhibitors such as ninhydrin N ethyl maleimide 2 4 dinitrophenol and thyroxine

In rat mast cell granules the histamine is electrostatically linked to carboxyl groups in the protein heparin complex and the histamine is instantaneously released on exposure of the granules to cation-containing media (Åborg Novotny and Uvnäs 1967)

It was proposed from this laboratory (*Thon and Uvnäs 1967*) that the histamine release induced by compound *48/80* occurred as a two stage response the first step—the degranulation being an energy requiring discharge of histamine containing granules from the mast cells the second step the histamine release being an extracellular physicochemical process an ion exchange between histamine in the granules and cations predominantly sodium ions—in the extracellular medium

The present results are consistent with the assumption that d tubocurarine al

though a monomer, activates a similar two step release mechanism to that triggered by the polymer compound 48/80 Definite proof of this idea—the demonstration of the discharge of histamine containing granules—is beset with the difficulty that the d tubocurarine concentration needed to activate the degranulation mechanism is sufficient to result in a cationic exchange in the extruded granules between the bis-quaternary base and the histamine It remains to be shown, for example by using radioactively labelled d-tubocurarine, either that d tubocurarine does not enter the mast cells (as is most probably the case) or that, if it enters it does so in concentrations which will cause no significant liberation of histamine by intracellular cation exchange in the granules

Hitherto all agents known to trigger the degranulation of rat mast cells have been polymer amines, either biogenic polypeptides or synthetic amines It might be assumed that they attach to receptors on the cell surface, thereby triggering processes in the cell membrane which lead to the discharge of granules from the cell d-Tubocurarine is a bisquaternary ammonium base of known structure and a study of the histamine releasing properties of the many structurally-related synthetic derivatives might give a better insight into the mechanism of action of degranulating agents and into the relationship between chemical structure and degranulating activity

References

ABORG C H, N NOVOTNY and B UVNÄS Ionic Binding of Histamine in Mast Cell Granules *Acta physiol scand* 1967 *69* 276—283

ALAM, M, G V ANREP, G S BARSOUM M TALAAT and E WEININGER, Liberation of histamine from the skeletal muscle by curare *J Physiol* (Lond) 1939 *95* 148—158

ARCHER G T Release of histamine from the mast cells of the rat *Austr J exp Biol med Sci* 1959 *37* 333—337

COMROE, J H and R D DRIPPS The histamine like actions of curare and tubocurarine injected intra-cutaneously and intra arterially in man *Anesthesiology* 1946 *7* 260—265

MONGAR, J L and D WHELAN Histamine release by adrenaline and d tubocurarine in the human subject *J Physiol* (Lond) 1953 *120* 146—154

PATON, W D M Histamine release by compounds of simple chemical structure *Pharmacol Rev* 1957 269—328

ROCHA E SILVA M and H O SCHILD The releas [illegible] the [illegible] *[illegible]arm* [illegible] the isolated rat diaphragm *J Pharm Pharmacol* 1967 *19* 125

SHORE P, BURKHALTER, A and COHN, JR W A method for the fluorometric assay of histamine in tissues *J Pharmacol exp Ther* 1959 *127* 182—186

THON, I L and B UVNÄS Degranulation and histamine release Two consecutive steps in the response of rat mast cells to compound 48/80 *Acta physiol scand* 1967 *71* 303—315

UVNÄS B and I L THON Evidence for enzymatic histamine release from isolated rat mast cells *Exp Cell Res* 1961 *23* 45—57

Acta physiol. scand. 1969. 76. 340—351

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Production and Distribution of Intracranial and Intraspinal Pressure Changes at Sudden Extradural Fluid Volume Input in Rabbits

By

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Received [illegible] September 1968

Abstract

LINDGREN, S. and L. RINDER. *Production and distribution of intracranial and intraspinal pressure changes at sudden extradural fluid volume input in rabbits.* Acta physiol. scand. 1969. 76. 340—351.

A device is described for sudden application of a mechanical load to the brain in animals by displacing a column of fluid within a piston system towards its connection with a hole in the skull. Pressure and displacement of the piston forcing the fluid into the skull cavity were studied in a series of experiments in rabbits. Simultaneous recordings were made of the pressure pulses produced in the fluid near the parietal site of loading of the brain and at several places within the contents of the skull cavity and spinal canal. By adjusting the fluid input to the skull cavity it was possible to produce intracranial pressure pulses predictable with respect to peak amplitude and duration within a range between 0.1 and 4.0 atm and one and several hundred msec, respectively. The pressure pulses produced intracranially and in the major part of the skull content were similar. Simultaneous recordings of the pressure course in the region of the craniospinal junction and in the spinal canal revealed however that peak amplitude and duration of the pressure pulse then decreased continuously with increasing distance from the site of application of the load. No pressure changes were found to occur caudally to the level of the first thoracic vertebra. This method seems suitable for studying the effects of controlled and varied sudden mechanical loading of the brain in animals and the relations between subsequent intracranial pressure changes and pathological alterations similar to those occurring in skull trauma.

Effects on the central nervous system (CNS) produced by brief mechanical events within the contents of the skull varies due to the characteristics of the applied load. In studying relations between mechanical and pathological changes within the CNS useful information may be obtained from animal experiments with controlled and varied intracranial mechanical effects.

Sudden application of mechanical load to the brain through a hole in the skull (Duret 1878, Kocher 1901, Miller 1927, Denny-Brown and Russell 1941, Clark and Ward 1948, Gross et al. 1958, Walker et al. 1944, Eichelberger et al. 1949) pro-

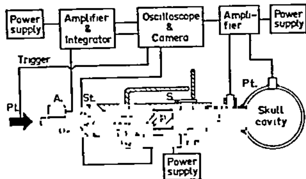


Fig 1 Schematic drawing of the experimental arrangement and block diagram of recording equipment Plunger system to the left and the connected animal skull to the right
Pl.=pendulum, A =accelerometer P r =piston rod, St = adjustable stopper, P =piston, S =shutter, P m =potentiometer, P t =pressure transducers

ducing a transient rise in intracranial pressure (Gurdjian *et al* 1954, Lindgren and Rinder 1966, 1967), has proved useful to elicit common pathophysiological signs of "experimental brain concussion" and pathoanatomical alterations similar to those observed following impacts on the intact animal skull Control and variation of such load applied to the brain may be comparatively easy to perform Study of subsequent pressure changes within the C.N S is of interest, because some information is available on intracranial pressure changes at a blow against human cadaver skulls (Lissner *et al* 1960, Sellier and Unterharnscheidt 1963, Lindgren 1966, Thomas *et al* 1967, Hodgson 1968), possible relations between pressure differences and injury produced at the cranio-spinal junction have been frequently discussed

The present report describes an equipment for sudden application of a mechanical load to the brain in animals by displacing a column of fluid into the skull cavity through a hole in the skull The usefulness of this method to produce graded and reproducible loading was studied in experiments in rabbits and the subsequent pressure pulses were recorded simultaneously at various sites in the skull cavity and spinal canal

Material and methods

Animals and preparations

30 rabbits, weighing 2.5—3.5 kg were used Anesthesia was induced and maintained by i.v. injection of 6% sodium mebumal solution Some experiments were performed on animals sacrificed by administration of an over-dose of the anesthetic immediately before the experiment In the living animals local anesthesia was used in the operative fields

A parietal hole (diam 1.5 mm) was made in the midline with a hand trephine the dura mater was left intact Bone edges were smoothened and bone surfaces around the hole were exposed and dried A plexiglass tube (inner diam. 16 mm length 25 mm Fig 1—3) provided with an end collar was inserted into the trephined hole until it just reached the dura The tube was fixed to the surrounding bone with quick hardening plastic solution (Simplex Rapid® Dental Fillings Co England) and to screws in the frontal and occipital skull bones The fixation thus occurred through a comparatively thick plastic layer "solded" to the screws and the skull bones even allowing heavy oblique external load to the tube

One to three further holes (diam 6 mm) were made in various parts of the skull bones and spinal column (Fig 3) for the insertion of pressure transducers

Equipment for application of graded load to the brain

A metal plunger system was designed in such a way that its open end could be tightly connected with the plexiglass tube fixed in the trephined hole (Fig 1—2)

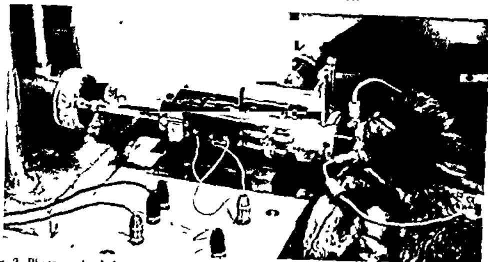


Fig 2 Photograph of the experimental arrangement (cf Fig 1) Pendulum — plunger system — rabbit skull

plunger system and the connected plexiglass tube were completely filled with physiological saline of room [illegible]

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The movement of the piston was arrested by an adjustable collar (stopper) on the piston-rod (Fig 1—2) The pressure course in the plunger system could be interrupted by opening of a shutter in the cylinder wall by the impact of an adjustable lever from the piston rod (Fig 1—2)

Recording equipment

A precision *potentiometer* (RLP 25, Swema, Sweden) was rigidly fixed to the wall of the plunger system and connected with the piston-rod (Fig 1—2) The signal from the potentiometer was recorded on an oscilloscope The potentiometer displacement was calibrated by moving [illegible]

An *acc*[illegible] k, nat freq 47 kHz) was fixed to the piston-rod [illegible] integrated to velocity or displacement recording [illegible] oscilloscope Calibration was performed at 1 g [illegible]

Piezoelectric miniature pressure transducers (Vibrometer 6 01, Switzerland diam 6 mm, nat freq 150 kHz sensitiv 8 pC/kp/cm²) were inserted laterally into the plexiglass tube perpendicular to the impact direction near the brain surface ("extracranially") and in the trephined holes in the skull and spinal canal flush with the dura (Fig 1—3) The latter trans



Fig 3 Sagittal mid line section through the skull and upper spinal canal of a rabbit with a medullary lesion after an experiment Location of trephined hole with marks for tube insertion parietally and numbers for location of pressure transducers 1—9

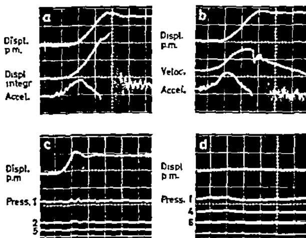


Fig 4 a—b Oscilloscope recordings of piston movement at impact on the piston rod by the pendulum
c—d Control of pressure transducer acceleration sensitivity during experimental conditions Piston rod impacted with no fluid (c) in the plunger system and with fluid and the piston rod rigidly fixed (d)
Piston displacement *displ p m* (recorded from potentiometer), *displ integr* (from accelerometer) —Piston velocity *veloc* (obtained from accelerometer) —Piston acceleration *accel* —Pressure *press*, recorded from pressure transducers in the locations shown in Fig 3
Calibrations Piston displacement 0.9 mm/square, acceleration 15 g/square, pressure 1 2 atm / square, time 2 msec/square

ducers were fixed to the surrounding bone with the plastic solution In some experiments a smaller pressure transducer (LC-5, Atlantic Research Corp, U S A, diam 2 5 mm) was inserted

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oscilloscope was triggered by the contact between the pendulum and the piston rod The record

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animal, connected with the loading equipment was placed on a table with the X-ray beam parallell to the plane through the end surface of the tube with brain surface/contrast fluid boundary clearly reproduced The X ray screen was photographed with a movie camera at about 80 exposures/sec exposure time was about 3 msec The impact of the pendulum was marked with a light flash on the film strip

Control of experimental procedures

The *velocity of the pendulum* at impact on the piston rod was studied by photographing the fall of the pendulum with a stroboscopic light source Variation of impact velocity, at constant fall height was found to be less than 10 per cent

The *accelerometer* was mounted with its axis of main sensitivity in the direction of piston movement with a screw on the piston rod (Fig 1—2) Influence of cross sensitivity and mode of mounting was studied with the accelerometer mounted with its main axis perpendicular to the main impact axis. At piston impact only minor high frequent deflections of the accelerometer signal were recorded At the arrest of the piston by the stopper the accelerometer displayed signals of high frequency and amplitude (Fig 4 a, b), these signals were used as an indication of time of arrest of piston movement The pressure increase in the plunger system during loading of the brain could be suddenly interrupted by opening of a shutter at impact of a lever connected with the piston rod This impact produced a high frequent oscillation of the accelerometer signal which was used to determine the moment of opening of the shutter

The distance and time course of the movement of the piston was measured with the *potentiometer* connected with the piston rod These recordings were compared with the

recordings of velocity and displacement of the piston rod, obtained by electronic integration of the accelerometer signal (Fig 4 a, b) They showed some latency of the start of the potentiometer recording and a "hunch" and delay at the arrest of piston movement This lag may be due to elasticity of the connection between the piston rod and the potentiometer It was however considered that displacement and "mean" velocity (calculated from distance and duration of movement) of the piston could be obtained from the potentiometer recordings with satisfactory accuracy

The effect of displacement of the piston and the fluid is partly dependent on the *tightness* of the system The open end of the plexiglass tube [illegible]

[illegible]

foil was produced The volume of the plexiglass tube was determined again and the deformation *of the lead foil was calculated from the difference between the volume of the plexiglass* tube before and after deformation of the foil Differences between the amount of fluid displaced, obtained from the potentiometer recording and from the deformation of the foil respectively, were found to be less than 10 per cent, and it was considered that leakage of fluid during the loading of the brain was not an error of major importance — Leakage of fluid between the end of the plexiglass tube and the plastic seal around the trephined hole was controlled visually and was also checked at the X-ray cinematography of the contrast filled plunger system.

The piezoelectric *pressure transducers* have high natural frequency suitable for measurements of rapid pressure transients. They are however sensitive to acceleration (0 002 atm/g according to the manufacturer) which may result in considerable errors of pressure recordings made during impact conditions (cf Lindgren and Rinder 1965) To study the influence of this factor in the present experiments the piston rod was impacted under varying conditions with the piston rod rigidly fixed by the stopper, with no fluid in the plunger system and with the pressure sensitive membrane of the transducer protected by an air filled case In these control experiments there were only minor deflections in the pressure recordings (Fig 4 c d)

Results

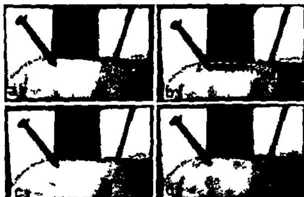
Piston movement and extracranial pressure course

The displacement of the piston from the start at the impact by the pendulum to the arrest at the contact between the stopper and the cylinder was varied between 0 2 and 5 mm corresponding to a calculated fluid volume displacement of 0 04 to 1 ml The mean velocity of the piston was varied between 0 05 and 2 m/sec This resulted in a gradual increase of the pressure recorded within the plunger system which reached a peak value at the arrest of the piston between 0 1 and 4 0 atm With known displacement and mean velocity of the piston the amplitude and duration of the pressure pulse produced could be predicted and repeated with good accuracy (*cf Fig 7*) *Errors in the experimental procedures e g leakage of fluid* during the loading could thus be revealed The duration of the pressure pulse was varied between 1 and 300 msec and its later course could be interrupted by opening of the shutter

The signal from the piston-rod accelerometer usually had the shape of a half sine wave (Fig 4 a, b), interrupted by high frequency disturbances at the impact of the stopper against the wall of the plunger Peak amplitude of the acceleration pulse seldom exceeded about 25 *g* Piston velocity seemed to be fairly constant in the later part of the piston movement also with comparatively short pulses Piston displacement, obtained by integration of the accelerometer signal showed a course resembling the smooth rise of the potentiometer recording (Fig 4 a)

Fig 5 Four consecutive exposures of the fluid/brain surface boundary during an X ray cinematography experiment Impact on the piston rod by the pendulum occurs at exposure *b* indentation of the brain surface which disappears in exposures *c* and *d* — The piston was displaced 1 5 mm at a mean velocity of about 0 5 m/sec The shutter opened at the arrest of piston movement Exposure rate about 80/sec and exposure time 3 msec

Occipital region to the left in the X rays



During the movement of the piston the pressure recorded within the plunger system (no 1 in Fig 3) showed a continuous rise, which was suddenly interrupted at the moment of arrest of the piston irrespective of whether the shutter was opened or not With increasing mean velocity of the piston the rate of pressure rise also increased (*cf* Fig 7) If the shutter in the plunger wall was opened at the arrest of piston movement pressure fell suddenly to zero (Fig 6 b) With the shutter still closed an immediate pressure fall was later followed by more gradual decrease to zero (Fig 6 a) The shape of the pressure pulses produced was thus approximately triangular

Brain surface displacement Pressure course in skull cavity and spinal canal

In the X ray cinematography experiments exposures were made at a rather slow rate compared with the time course of piston movement However the film strips clearly showed that no contrast fluid was displaced laterally between the dura and the skull bones but the brain surface under the trephined hole was indented locally by the fluid input The deepest indentation was not situated under the center of the trephined hole but in the occipital direction (Fig 5 b) The brain surface was permanently indented until the shutter was opened later When the shutter was opened at the end of piston movement the indentation was no longer seen on following exposures (Fig 5 d)

The pressure pulses recorded from transducers located in the frontal and occipital skull holes (nos 2 and 3 Fig 3) were always similar to the one obtained simultaneously from the extracranial transducer (Fig 6 and 8) The levels of peak pressure recorded in these locations were thus equal with that obtained extracranially It was difficult in the routine experiments to determine time differences between the start of the pressure pulses as the pressure rise was comparatively slow at the beginning of piston movement There was however a slight delay of the peaks of the pressure pulses recorded in the frontal and occipital locations compared with the pressure pulse recorded within the plunger system (Fig 6 and 8)

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The effect of displacement of the piston and the fluid is partly dependent on the *tightness* of the system. The open end of the plexiglass tube (to be inserted into the trephined hole) supplied with the pressure transducer, was covered with a piece of lead foil, and the volume of the tube was determined by weighing its fluid contents By impacting the piston rod the contents of the tube was then displaced a short distance and a permanent deformation of the lead foil was produced The volume of the plexiglass tube was determined again and the deformation of the lead foil was calculated from the difference between the volume of the plexiglass tube before and after deformation of the foil Differences between the amount of fluid displaced obtained from the potentiometer recording and from the deformation of the foil, respectively, were found to be less than 10 per cent, and it was considered that leakage of fluid during the loading of the brain was not an error of major importance — Leakage of fluid between the end of the plexiglass tube and the plastic seal around the trephined hole was controlled visually and was also checked at the X ray cinematography of the contrast filled plunger system

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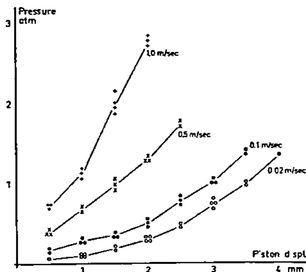


Fig 7 Relations between peak amplitude of the extracranial pressure pulse (no 1 in Fig 3) and displacement of the piston moving the fluid into the skull cavity at varying mean piston velocity

Discussion

Reproducible conditions and reliable measurements of intracranial mechanical effects are more difficult to obtain with impact to the animal skull than with direct loading of the brain (*cf* Gurdjian *et al* 1965 Lindgren and Rinder 1965) Effects of comparatively slow application of such load, *e g* inflation of an intracranial extradural balloon, have been frequently studied, but there are few investigations within the higher frequency range and often with no report concerning input magnitude and course

Gurdjian *et al* (1954) used application of compressed air to the exposed parietal dura in dogs There was a steep rise in intracranial pressure (6—74 psi[1]) lasting between 1 and 100 msec, with production of high levels of intracranial pressure, however there were marked oscillations in the pressure recordings and an average level had to be determined —Fluid loading may produce more easily controlled and varied intracranial pressure effects and cause less contact phenomena and local shearing effects than loading with a solid object (v Gierke 1966)

In this study the control experiments seemed to show that there were no considerable errors in the piston displacement and pressure recordings Other experiments performed to control the possibility of fluid leakage during the procedures seemed to show that the system could be made sufficiently tight, knowledge of the routinely obtained pressure recording from the extracranial transducer was useful to reveal the occurrence of leakage of fluid during the loading of the brain (*cf* Fig 7)

The mean velocity and displacement of the piston moving the fluid column extradurally into the skull cavity could be adjusted and recorded In Fig 7 it is demon

[1] psi pounds per square inch 14 7 psi = 1 atm

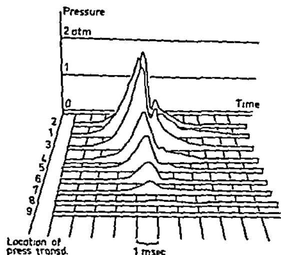


Fig 8 Drawing of pressure pulses recorded at various sites (cf Fig 3) in the skull cavity and cervical spinal canal at experiments with similar loading of the parietal brain surface

strated that the extracranial peak pressure level is related to both volume and rate of the fluid displaced in the plunger—skull system The fluid movement into the skull cavity was not possible to measure directly, X-ray cinematography experiments showed indentation of the brain surface underneath the parietal hole The course of this deformation could not be studied, due to the comparatively slow closure rate, but the film strips seemed to show that the main part of the indentation occurred in the occipital part and not in the center of the trephined hole (Fig 5b) indicating the direction of maximum tissue deformation. At high rate loading some further deformation of the brain may occur after the arrest of the piston

The fluid volume input and the pressure increase built up extradurally may exert full effect on the exposed dura resulting in the intracranial pressure peak at the arrest of the piston The dural sac with the brain and spinal cord constitutes a complex mechanical system and its response to different mechanical inputs varies (cf Goldsmith 1966) In the present experiments loading time was long compared with the apparent velocity of propagation of the pressure pulse and the pressure course produced extracranially within the fluid column and in the major part of the contents of the skull cavity were found to be almost identical with respect to pressure rise, peak amplitude and duration

Controlled and varied sudden mechanical loading of the brain with a similar extra- and intracranial pressure pulse, could be produced with the present equipment Thus in studying correlations between pathophysiological and morphological effects and intracranial pressure course at such loading insertion of the pressure transducer into the skull contents will not be required and artifacts due to procedures for intracranial pressure measurements can be avoided

The course of displacement of the contents of the skull cavity and spinal canal produced by the fluid volume input may be related to differences in magnitude

time lapse and duration of the pressure course at various sites Amplitude and duration of the pressure pulse gradually decreased and increasing pulse delay occurred at the cranio spinal junction and the upper part of the spinal canal —The results of experiments performed by Gurdjian *et al* (1967) and Hodgson (1968) are suggestive of suddenly occurring displacement of the tissues at the cranio spinal junction during and after the end of loading of the brain They introduced lead tags via the foramen magnum into the brain stem in dogs Flash X ray recordings were made both during impact to the intact skull and on application of compressed air to the exposed dura At a hammer blow against the parietal part of the fixed skull of a dog which seemed to produce a local indentation at the impact site there was some deformation of the tags in the brain stem area with its convexity towards the spinal canal suggesting that a flow of cerebral tissue towards the foramen magnum is in process The tags returned to their normal position and it was concluded that the deformation of the brain was elastic In experiments with an impact in the mid occipital area to the free head of a monkey the pellets in the brain also moved towards the foramen magnum After application of a 20 psi and 20 msec duration air pressure pulse to the exposed dura the lead tags apparently moved towards the spinal cord but might return to the pre injury position The application of 30 psi increment resulted in the death of the animal and the tags did not return to the pre impact position —In similar experiments, with sudden application of a mechanical load to the exposed dura patho-anatomical alterations have been produced and mainly confined to the brain stem and upper cervical cord (Chason *et al* 1967 Lindgren and Rinder 1967 Rinder and Olsson 1968) Application of a sudden load to the parietal part of the brain surface in the rabbit produces displacement of brain tissue through a frontally or occipitally located decompressive hole (dura removed where a marked pressure difference must be present the damage of the brain stem and cervical cord was less than after application of the same load without decompression of the brain (Lindgren and Rinder 1967) —These results support the assumption that displacement and deformation of the tissues at the cranio-spinal junction occur and are important mechanical effects in producing damage in these areas in such experiments

Pressure changes of a few msec duration occur within the contents of the skull on impacting human cadaver skulls (Lissner *et al* 1960 Sellier and Unterharnscheidt 1963 Lindgren 1966 Thomas *et al* 1967 Hodgson 1968) If there is no fracture intracranial pressure pattern will vary mainly according to acceleration and velocity differences between the skull and its contents and a transient pressure gradient will occur along the main axis of movement of the head with a positive pressure area at the impact pole and a negative pressure area on the other side If a blow produces a large depressed fracture there will be a temporary rather uniform and comparatively slow increase of intracranial pressure within the whole skull i e similar with the pressure course produced in the present experiments At impacts on a child s skull with comparatively low rigidity the accelera

tion pressure pattern is also less marked (Lindgren 1966) At impacts to skulls without fracturing change in volume of the skull may also occur Pressure differences between the skull cavity and upper part of the spinal canal have been found to occur in such experiments (Lindgren 1966), and may cause tissue displacement in these areas In the present study pressure changes at the cranio spinal junction were of particular interest —Results of experiments on closed skull models also point to the occurrence of mechanical effects in this region at skull impact Gurdjian and Lissner (1961) and Edberg *et al* (1963) observed shear lines predominantly at the simulated cranio-spinal junction following impacts on skull artifacts filled with photoelastic material High speed cinematography of the junction between a duralumінium sphere and a plexiglass "spinal canal' revealed an outward displacement of the layer between two fluids within a few msec following a blow against the 'occipital' pole of the sphere (Lindgren *et al*) The results of the flash X-ray experiments performed by Gurdjian *et al* and Hodgson (see above) are also suggestive of such effects of a blow on the intact animal skull

The present method produces controlled and varied mechanical loading of the brain in animal experiments, with predictable pressure changes within the contents of the skull cavity and spinal canal It may be valuable in elucidating relations between intracranial mechanical events and pathological effects produced

We are indepted to Dr Bengt Zachrisson Roentgen Department I, University of Goteborg for kind cooperation in the X ray cinematography experiments

The investigation has been supported by grants from the Medical Faculty of the University of Goteborg Statens Trafiksakerhetsråd and Trygg Fylgias Attioårsfond

References

CHASON J L B F HADDAD J E WEBSTER and E S GURDJIAN Alterations in cell structure following sudden increases in intracranial pressure *J Neuropath exp Neurol* 1957 *16* 102—107

CLARK S L and J W WARD The effect of rapid compression waves on animals submerged in water *Surg Gynec Obstet* 1943 *77* 403—412

DENNY BROWN D and W R RUSSELL Experimental cerebral concussion *Brain* 1941 *64* 93—164

DURET H *Études experimentales et clinique sur les traumatismes cerebraux* These de Paris 1878

EDBERG S J RIEKER and A ANGRIST Study of impact pressure and acceleration in plastic skull models *Lab Invest* 1963 *12* 1305—1311

EICHELBERGER L J KOLLROS and A WALKER Water nitrogen and electrolyte content of brain following cerebral concussion *Amer J Physiol* 1949 *156* 129 136

VON GIERKE H E On the dynamics of some head injury mechanisms In *Head Injury Conf Proc* Ed W F Caveness and A E Walker pp 383 396 J B Lippincott Co Philadelphia Toronto 1966

GOLDSMITH W The physical processes producing head injuries In *Head Injury Conf Proc Ed W F Caveness and A E Walker pp* 350—382 J B Lippincott Co Philadelphia Toronto 1966

GROAT, R A, H W MAGOUN F L DEY and W F WINDLE Functional alterations on motor and supranuclear mechanisms in experimental concussion *Amer J Physiol* 1944 *141* 117—127

GURDJIAN E S and I ... strains at the craniospinal ... Studies

GURDJIAN E S, H I ... acranial on experimental concussion Relation of physiological effect to time duration of ... pressure increase at impact *Neurology* (Minneap) 1954 *4* 674—681

GURDJIAN, E S, V R HODGSON, L M THOMAS and L M PATRICK, High speed techniques ın head injury research Demonstration of relative movements of scalp, skull and intracranial contents during impact *Med Science* 1967 *18* 45—56

GURDJIAN, E S, H R LISSNER and L M PATRICK, Concussion—mechanism and pathology In *Proc 7th Stapp Car Crash Conf* Ed D M Severy pp 470—482 C. C Thomas Publ Springfield, Ill 1965

HODGSON, V R, *Head impact response of several mammals including the human cadaver* [illegible]

LINDGREN, S, Experimental studies of mechanical effects in head injury *Acta chir scand* 1966 Suppl 360

LINDGREN, S and L RINDER, Experimental studies ın head injury I Some factors influencing results of model experiments *Bıophysık* 1965 *2* 320—329

LINDGREN, S and L RINDER, Experimental studies ın head injury II Pressure propagation ın "percussion-concussion" *Bıophysık* 1966 *3* 174—180

LINDGREN, S and L RINDER, Decompression ın percussion-concussion effects on "concussive response" ın rabbits *J Trauma* 1967 *7* 493—499

LINDGREN, S, L RINDER and B ÅSBERG, Unpublished experiments

LISSNER, H R, M LEBOW and F G EVANS, Experimental studies on the relation between acceleration and intracranial pressure changes ın man *Surg Gynec Obstet* 1960 *111* 329—338

MILLER, G G, Cerebral concussion *Arch Surg* 1927 *14* 891—916

RINDER, L and Y OLSSON, Studies on vascular permeability changes ın experimental brain concussion 1 Distribution of circulating fluorescent indicators ın brain and cervical cord after sudden m [illegible]

SELLIER, K and [illegible] nach stumpfer [illegible]

THOMAS, R K, [illegible] three orthogon [illegible]

WALKER, A E, [illegible] *Neurosurg* 1944 *1* 103—116

Acta physiol. scand. 1969. 76. 352—360

From the Department of Hygiene, University of Goteborg, Sweden

"Concussive Response" and Intracranial Pressure Changes at Sudden Extradural Fluid Volume Input in Rabbits

By

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Received 19 September 1968

Abstract

RINDER, L "Concussive response" and intracranial pressure changes at sudden extradural fluid volume input in rabbits Acta physiol scand 1969 76 352—360

An extradural fluid volume (0 04—1 0 ml) was suddenly applied to the parietal brain surface in rabbits by means of a plunger system, attached to a hole in the skull The immediate changes in arterial blood pressure, pulse rate and respiration were recorded and used as criteria of a "concussive response" These pathophysiological effects were related to peak amplitude (0 1—3 0 atm) and duration (5—300 msec) of the recorded intracranial pressure pulse Similar pathophysiological effects could be reproduced by application of the same single load to the brain through a series of experiments The "severity" of the concussive response varied with the peak amplitude and duration of the intracranial pressure pulse At pressure pulses of 5—15 msec duration no pathophysiological effects occurred with a pressure pulse not exceeding about 0 7 atm peak amplitude, but at 2 5 atm peak pressure irreversible apnea was produced Decrease in blood pressure occurred at induction of pressure pulses of comparatively low magnitude while a sudden increase in blood pressure was produced at pressure pulses of higher magnitude Duration of apnea increased with increasing peak amplitude of the pressure pulse and seems to be a useful measure of the "severity" of the concussive response in the present type of brain trauma

At head injuries some of the immediately occurring pathophysiological effects seem to be elicited from brain stem areas (*cf* Ward 1958 1966) A blow to the head in animals may also produce such immediate vasomotor respiratory and reflex disturbances, generally used as criteria of "experimental brain concussion (*cf* Denny-Brown and Russell 1941 Denny-Brown 1945 Gurdjian *et al* 1955 Ommaya 1966 a Hodgson 1968) Recent investigations suggest that the brain stem is probably displaced towards the spinal canal at impacts on the animal skull (Gurdjian *et al* 1967, Hodgson 1968) and the occurrence of cranio spinal pressure differences may indicate similar effects at a blow on human cadaver skulls (Lindgren 1966 Thomas *et al* 1967)

At sudden mechanical loading of the brain through a hole in the animal skull, producing a steep rise in intracranial pressure, the brain stem may be displaced towards the spinal canal (*cf* Rinder and Lindgren 1969) and a "concussive response"

may occur (*cf* Denny Brown 1945, Gurdjian *et al* 1954, Lindgren and Rinder 1966 1967) With such loading controlled experimental conditions can be obtained avoiding more complex mechanical effects from a blow to the intact skull There are few reports on relations between load input course intracranial pressure variations and pathophysiological effects produced at such sudden mechanical loading of the brain

A previous report (Rinder and Lindgren 1969) described an equipment for sudden extradural application of fluid volume load to the brain in animals It was possible to produce predictable pressure pulses of varying peak amplitude and duration, resembling those recorded in human cadaver head impact experiments (Lindgren 1966, Thomas *et al* 1967, Hodgson 1968) Marked pressure differences occurred at the cranio-spinal junction —In the present investigation the production of some immediate pathophysiological effects, generally used as criteria of experimental brain concussion were recorded following application of load to the brain in rabbits, the effects were related to peak amplitude and duration of the intracranial pressure changes

Material and methods

Animals and preparations

155 rabbits of both sexes weighing 2 3—3 3 kg were used Anesthesia was induced (0 5 ml/kg b w) and if necessary maintained (0 2—0 3 ml) by *i v* injection of 6 % sodium mebumal solution (Mebumal® ACO Sweden) Local anesthetic (Xylocain®, Astra Sweden) was used in operative fields The interval between induction of anesthesia and application of load to the brain was about 100 min

A hole (diam 17 5 mm) was made in the mid line of the exposed parietal skull bone and a well fitting plexiglass tube was inserted and fixed to the skull as described previously (Rinder and Lindgren 1969) The dura was mostly kept macroscopically intact

A polyethylene tube (PE 160) was inserted into the femoral artery and advanced up into the abdominal aorta for continuous recordings of arterial blood pressure and pulse rate

Equipment for application of load to the brain Recording of piston displacement (fluid input) and subsequent pressure pulse

The equipment used has been described in detail previously (Rinder and Lindgren 1969) Thus a plunger system with the connected plexiglass tube was completely filled with physiological saline of room temperature The piston of the plunger system was moved towards the brain surface by the impact of a pendulum or by means or a lever A pressure transducer was inserted into the plexiglass tube near the exposed brain surface The pressure pulse measured at this site at the loading of the brain was similar in rise time peak amplitude and duration with the pressure pulse recorded simultaneously within major part of the skull cavity — At the arrest of the piston moving the fluid extradurally into the skull cavity, a shutter in the cylinder could be opened thus releasing the pressure (Fig 1) and the indentation of the brain surface disappeared within a few msec The movement of the piston and the pressure pulse produced were recorded simultaneously on an oscilloscope (Fig 1) —Calibration of the pressure transducer was performed as described previously (Rinder and Lindgren 1969)

Recordings of pathophysiological effects and experimental procedure

Arterial blood pressure was measured by connecting the tube inserted into the femoral artery with a strain gage pressure dome (Statham T-23 U S A) The signal was amplified (Honeywell 131 2 C U S A) and recorded on an UV recorder (Visicorder, Honeywell) Calibration was performed with the aid of the original electronic calibration unit and was checked by connecting the pressure dome with a mercury column of varying height

Pulse rate could be obtained from the blood pressure recording or—for practical purposes —by connecting a signal output from the blood pressure amplifier with a heart rate meter

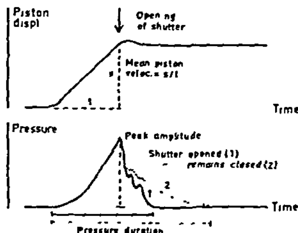


Fig 1 Schematic drawing of oscilloscope recording routinely obtained at application of the load to the brain showing *displacement of piston* and the simultaneous *pressure pulse* measured with a transducer inserted extracranially near the site of loading of the brain *Mean piston velocity* was calculated from distance of piston movement (s) and the time from start to arrest of piston movement (t) When the shutter was opened at the arrest of the piston the pressure decreased suddenly to zero (1), when the shutter remained closed pressure later decreased more slowly (2)

(EMT 50 Elema Schonander Sweden) This was supplied with a specially designed preamplifier made to count but every second peak of the arterial pulse waves The pulse rate signal thus obtained was recorded on the Visicorder The unit was calibrated with a pulse generator It was possible to record the pulse rate within a range of 30—400 beats/min and a change in pulse rate of about 20 beats/sec

Respiratory movements were recorded by a mercury filled rubber tube applied around the chest of the rabbit (Yellow Springs Instr Corp U S A) and connected with an amplifier (Honeywell chest displacement gage control module) the signal was recorded on the Visicorder

The Visicorder was started 2—3 min before application of the load to the brain and continuously run for 10—15 min

The *corneal reflex* was tested immediately before application of the load to the brain The duration of subsequent loss of the corneal reflex usually was noted however *convulsions* with closure of the lids occurred at application of the load It was controlled that the animal reacted to painful stimuli such as hard tail pinch immediately before induction of the intracranial pressure pulse.

In most experiments only a single load was applied to the brain and if lethal effects were not produced the animals were sacrified at varying intervals However if the applied load did not produce pathophysiological effects sometimes another load of higher magnitude was applied. The brain and spinal cord were removed and examined for changes in cerebrospinal vascular permeability Rinder and Olsson 1968

Control experiments

The impact of the pendulum on the piston rod produces not only movement of fluid into the skull cavity but also other mechanical effects such as vibrations possibly transmitted to the attached head of the rabbit (*cf* Rinder and Lindgren 1969 To control the influence of such factors as well as of the preparation on the physiological parameters recorded the piston rod was impacted without fluid in the plunger system and with fluid but with the piston rod rigidly fixed so that there was no displacement of the piston at the impact of the pendulum. In these experiments no changes occurred in the recordings of blood pressure pulse rate and respiration and there were no effects on the corneal reflex and no convulsions.

Results

Pressure pulse duration 5—15 msec

In these experiments the piston was moved by an impact of the pendulum The mean velocity of the piston during the loading of the brain was about 1 m sec and the piston displacement, from start to arrest was varied between 0 2 and 2 0 mm. The fluid volume thus displaced extradurally into the skull cavity was calculated to

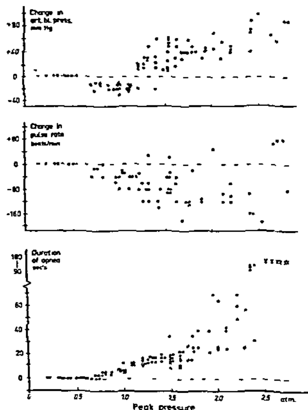


Fig 2 Results of experiments with induction of pressure pulses of 5—15 msec duration The piston was displaced 0 2—2 0 mm at a mean velocity of 1 m/sec Maximum initial changes (obtained within 2—15 sec after application of the load to the brain) in systolic arterial blood pressure and pulse rate and duration of apnea have been plotted against peak amplitude of the pressure pulse produced

○=shutter opened at arrest of piston (5—8 msec pressure duration),

●=shutter remained closed (12—15 msec pressure duration),

×=lasting apnea produced

vary between 0 04 and 0 4 ml In about half of the number of experiments the shutter opened at the arrest of the piston, the duration of the pressure pulses thus produced extra- and intracranially was about 5—8 msec In the other experiments the shutter remained closed at the arrest of the piston and was not opened until several sec later the pressure pulse duration was about 12—15 msec (*cf* Fig 1)

Application of load producing an intracranial pressure pulse with a peak amplitude not exceeding about 0 7 atm did not result in changes in the physiological parameters recorded With increasing peak amplitude immediate but transient effects occurred and at a peak amplitude exceeding about 2 5 atm irreversible apnea invariably occurred and heart activity ceased within 5—10 min. The effects produced and their relations to the peak amplitude of the intracranial pressure pulse were not dependent on whether the shutter opened at the arrest of the piston or not

Maximum levels of the changes in arterial blood pressure (BP) and pulse rate (PR) always occurred within 2—15 sec after application of the load to the brain and have been plotted against the peak amplitude of the intracranial pressure pulse together with the duration of apnea produced in Fig 2 With a peak amplitude

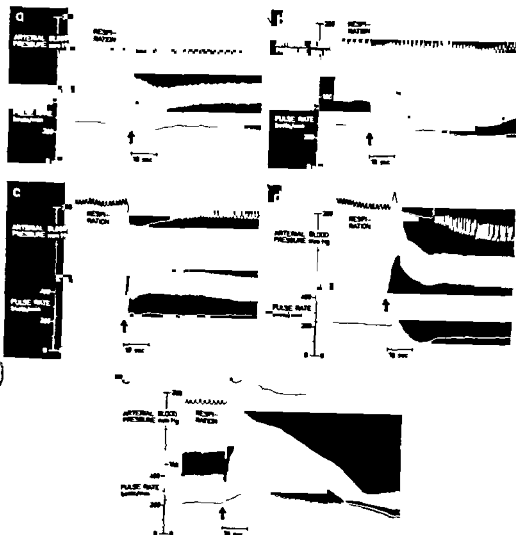


Fig. 3. Recordings of arterial blood pressure, pulse rate and respiration at induction of various pressure pulses by sudden application of a fluid volume to the brain. *Arrow* denotes application of load: a, peak pressure amplitude 0.7 atm., 5 msec duration; b, 1.0 atm., 12 msec; c, 1.3 atm., 6 msec; d, 1.8 atm., 14 msec; e, 2.6 atm., 8 msec.

of the intracranial pressure pulse in the range between 0.7 and 1.1 atm. there was always a decrease in BP during 20—60 sec (Fig. 3 a, b). Mostly PR was lowered during a somewhat shorter period. Respiratory arrest lasting 2—12 sec occurred; on return the rate and amplitude of chest displacement were usually similar to that recorded previous to the application of the load. When considered possible to evaluate, the corneal reflex mostly seemed to be unaffected; if lost its absence did not exceed 2 min. Often a sudden jerk occurred at the application of the load to the brain.

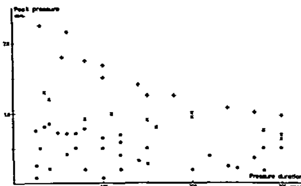


Fig 4 Results of experiments with induction of pressure pulses of 25—300 msec duration showing relations between "severity" of the concussive response and peak amplitude and duration of the pressure pulse The piston was displaced 1—5 mm at a mean velocity of 0 05—0 2 m/sec ○—no effects ●—"light" effect ×= moderate effect +="severe" effect (criteria are stated in "Results" p 6 line 6 from bottom)

With intracranial pressure pulses of more than 1 2 atm peak amplitude there was always (except for one experiment) a sudden rise in BP, however sometimes preceeded by a very transient fall (Fig 3 c d) BP then slowly decreased during 30—120 sec to the pre-existing level PR usually decreased during the same period but in some cases tachycardia was recorded (Fig 2) Duration of apnea was increased compared with experiments with pressure pulses of lower magnitude After the period of respiratory arrest the amplitude and rate of respiratory movements usually increased during 2—10 min (Fig 3 d) When considered possible to evaluate the corneal reflex was found to be absent for 1—30 min. A sudden jerk occurred at the application of the load to the brain followed by sudden flexion of the limbs during a few sec and later followed by relaxation

Pressure pulses exceeding 2 5 atm peak amplitude were always followed by irreversible apnea and sudden increase in BP falling to zero during the next 5—10 min Brady or tachycardia occurred (Fig 3 e)

Pressure pulse duration 25—300 msec

In these experiments the piston was moved at a mean velocity between 0 05 and 0 2 m/sec The piston displacement was varied between 0 5 and 5 0 mm corresponding to a calculated fluid input of 0 1 to 1 0 ml The shutter always opened at the arrest of the piston

The pathophysiological effects produced in these experiments were quite similar with those occurring on induction of pressure pulses of 5—15 msec duration, but the relations to the peak amplitude varied with the duration of the pressure pulse — In Fig 4 the severity of the pathophysiological effects has been plotted against peak amplitude and duration of the pressure pulse Thus a light concussive response was considered to have occurred when there was a decrease in BP and apnea during 2—12 sec The occurrence of a rise in BP with respiratory arrest during 10—90 sec was considered as a moderate concussive effect and if there was a lasting apnea a "severe" concussive response was considered to have been produced

Discussion

The present experiments showed that similar immediate pathophysiological effects generally used as criteria of a concussive response, can be reproduced through a series of animal experiments by sudden application of the same single mechanical load to the brain and that the "severity" of these effects varied with the peak amplitude and duration of the intracranial pressure pulse produced. Similar pathophysiological effects have also been reported with comparatively slowly applied load to the brain ("compression-concussion", cf. Denny-Brown 1945). Gurdjian et al. (1954) produced a sudden rise in intracranial pressure (6—74 psi[1], 1—100 msec duration) by application of compressed air to the exposed parietal dura in dogs. On the basis of the physiological effects produced they classified the reaction as no concussion, threshold concussion (in many instances a fall in blood pressure but no respiratory arrest), moderate (steep rise in blood pressure and apnea of varying duration) and severe concussion (mortally wounded). However, with production of intracranial pressure pulses of similar level and duration a concussive response of varying severity occurred but there was "a definite trend that the shorter the time duration the higher the pressure necessary to result in a concussive effect", i.e. similar to the results obtained in the present experiments.

In the present experiments duration of respiratory arrest generally increased with increasing peak amplitude of the intracranial pressure pulse, the duration of apnea may indicate the "severity" of the concussive response in this type of mechanical brain trauma. Hodgson (1958), in head impact experiments in dogs and monkeys found increased duration of apnea with increasing impact force and Friede (1961) found that duration of respiratory arrest was related to maximum velocity of the head at impacts to the skull in cats.—A sudden decrease in arterial blood pressure lasting 20—60 sec. always occurred at induction of pressure pulses of comparatively low peak amplitude; with pressure pulses exceeding a certain peak amplitude a steep rise in blood pressure occurred and may indicate a more "severe" concussive response. In head impact experiments the appearance of decrease in blood pressure has been considered to indicate a "subconcussive" response (cf. Gurdjian et al. 1954, Ommaya 1966 a, Hodgson 1968). With very heavy head impacts producing irreversible apnea in the animal an immediate and lasting drop of blood pressure has occurred (Denny-Brown and Russell 1941, Ommaya 1966 a); this was not observed in the present experiments with application of load producing irreversible apnea.—Duration of loss of corneal reflex seems to be the most observed single factor used to grade the "severity" of the concussive response in animal experiments. It was however difficult to determine that duration in the present experiments; preparations necessary to attach the loading equipment and repeated reflex tests may have influenced the possibilities for reliable observations.

When correlating the recorded mechanical effects with the pathophysiological changes in the present experiments, it must be remembered that the increase of

[1] 14.7 psi (pounds per square inch) = 1 atm.

the pressure in the plunger system and of the indentation of the brain surface were interrupted at the arrest of the piston This first part of the mechanical course was easier to control than the later course and the same pathophysiological response occurred irrespective of whether the shutter was opened or not The velocity and displacement of the piston and the extradural pressure increase may exert full effect at the brain surface, resulting in the intracranial pressure peak, however, due to mechanisms modifying the pathophysiological effect full correlation with piston displacement cannot be expected The time course of movement and deformation of the parts of the cranio-spinal contents involved in the occurrence of the pathophysiological effects may be of importance, Rosenbluth (quoted by Ommaya 1966 b) has shown that if axons were tapped mechanically in order to produce an action potential, the electric effect seemed to be related to the rate of change of velocity of the mechanical tap —So far reliable measurements of tissue displacement and deformation within the cranio-spinal contents are difficult to perform, and only some tentative experiments have been made to study such mechanical events At impacting the intact skull in dogs and monkeys and at application of compressed air to the exposed dura in dogs, Gurdjian *et al* (1967) and Hodgson (1968), using flash X-ray cinematography, observed displacement of lead tags, inserted into the brain stem, towards the spinal canal The lead tags might return to the "pre impact" position In the present experiments marked pressure differences occurred at the cranio-spinal junction (Rinder and Lindgren 1969), indicating displacement of skull contents towards the spinal canal Similar pressure differences have been recorded at impacts on human cadaver skulls (Lindgren 1966) In experiments, similar to those presented here, brain tissue protruded through another "decompressive" hole in the rabbits skull (dura removed) and the concussive response was absent or less "severe" than following application of the same load with no decompression (Lindgren and Rinder 1967), this also indicates the importance of mechanical effects at the cranio-spinal junction

The mechanical events occurring within the cranio-spinal contents at skull trauma are evidently complex and may differ considerably due to the characteristics of the applied load The experimental study of relations between brief intracranial intra-spinal pressure changes and pathophysiological and pathoanatomical alterations within the central nervous system may yield information useful in the discussion concerning the nature and magnitude of mechanical processes involved in the production of brain damage in skull trauma. To correlate pathological effects with displacement and deformation of the cerebrospinal tissues further information is necessary

I am indepted to Docent Sten Lindgren Department of Neurosurgery University of Goteborg for valuable suggestions during the preparation of the manuscript.

The investigation has been supported by grants from the Medical Faculty of the University of Goteborg Statens Trafiksakerhetsråd and Tryggve Fylgias Attioårsfond

References

DENNY-BROWN, D, Cerebral concussion *Physiol Rev* 1945 *25* 296—325

DENNY-BROWN, D and W R RUSSELL, Experimental cerebral concussion *Brain* 1941 *64* 93—164

FRIEDE, R L, Experimental acceleration concussion *Arch Neurol* (Chic) 1961, *4* 449—462

GURDJIAN, E S, H R LISSNER, J E WEBSTER, F R LATIMER and B F HADDAD, Studies on experimental concussion Relation of physiological effect to time duration of intracranial pressure increase at impact *Neurology* (Minneap) 1954 *4* 674—681

GURDJIAN, E S, J E WEBSTER and H R LISSNER, Ob[illegible] concussion, contusion and laceration *Surg Gynec O*[illegible]

GURDJIAN, E S, V R HODGSON, L M THOMAS and I [illegible] in head injury research Demonstration of relative [illegible] cranial contents during impact *Med Science* 1967 [illegible]

HODGSON, V R, *Head impact response of several mammals including the human cadaver* Dissertation (in manuscript) Wayne State University, Detroit, Mich 1968

LINDGREN, S, Experimental studies of mechanical effects in head injury *Acta chir scand* 1966 Suppl 360

LINDGREN, S and L RINDER, Experimental studies in head injury II Pressure propagation in "percussion-concussion" *Biophysik* *1966* *3* *174—180*

LINDGREN, S and L RINDER, Decompression in percussion concussion effects on "concussive response" in rabbits *J Trauma* 1967 *7* 493—499

LINDGREN, S and L RINDER, Production and distribution of intracranial and intraspinal pressure changes at sudden extradural fluid volume input in rabbits *Acta physiol scand* 1969 *76* 340—351

OMMAYA, A K, Trauma to the nervous system *Ann roy Coll Surg Engl* 1966a *39* 317—347

OMMAYA A K, In *Head Injury Conf Proc* Ed W F Caveness and A E Walker, p 510 J B Lippincott Co Philadelphia, Toronto 1966b

RINDER, L and Y OLSSON, Studies on vascular permeability changes in experimental brain concussion I Distribution of circulating fluorescent indicators in brain and cervical cord after sudden mechanical loading of the brain *Acta Neuropath* (Berl) 1968 *11* 183—200

THOMAS, R K, V L ROBERTS and E S GURDJIAN, Impact induced pressure gradients along three orthogonal axe[illegible] 316—321

WARD, A A Physiological b[illegible] 138

WARD A A, The physiology [illegible] W F Caveness and A E Walker pp 202 [illegible] nto 1966

Acta physiol scand 1969. 76 361—368
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The Effect of Menthol on Taste Receptors

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Received 23 September 1968

Abstract

HELLEKANT, G, *The effect of menthol on taste receptors* Acta physiol scand 1969 *76* 361—368

The response to menthol and its effect on the excitability of the gustatory receptors were studied by recording the impulse activity in the chorda tympani nerve of the cat It was ob-

This effect on the receptors was related to concentration and length of the preceding menthol rinse It showed also variations with regard to stimulus concentration and subject

It has been clearly demonstrated by earlier authors that menthol affects the response of many receptors to stimulation Like several other drugs it enhances in small concentrations and depresses in larger concentrations or after extended exposure Electrophysiologically this effect of menthol was first demonstrated for the thermal receptors by Hensel and Zotterman (1951) and Dodt Skouby and Zotterman (1952) Similar effects of menthol were observed in the chemoreceptors of the carotid body by Landgren Skouby and Zotterman (1953) An increase of the sensitivity as well as a decrease after menthol exposure were reported by Skouby and Zilstorff Pedersen for the olfactory sense (1954) and the gustatory sense (1955) These last authors used psychophysical technique in their studies It is felt that there is a gap to be bridged between the observations obtained with electrophysiological technique in the non gustatory receptors (Hensel and Zotterman 1951 Dodt *et al* 1952, Landgren *et al* 1953) and those with psychophysical technique in the gustatory (Skouby and Zilstorff Pedersen 1955) by applying electrophysiological technique to gustatory receptors The present study was undertaken with this aim

Methods

9 cats were used in this study The dissecting and stimulating techniques were the same as described in a previous study (Hellekant 1965 a) except for a longer water rinse which recently shown to be important (Hellekant 1968) The solutions were mad which also was used as a contin

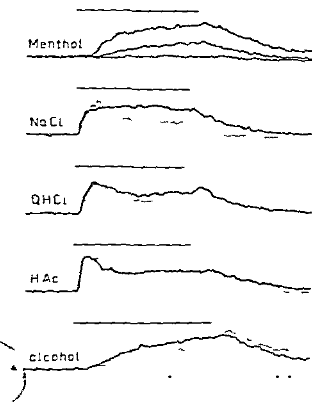


Fig. 1 shows a series of summated recordings from one cat. The top record displays the response to 0.05 g, 0.1 g and 0.2 g menthol/l water. The other records show the responses to 4 stimuli with 0.1 g menthol/l, dotted curve, and without menthol, unbroken curve. Time marker 1/sec.

the water flow was exchanged with a flow of menthol. Three concentrations of menthol were used, 0.05 g, 0.1 g and 0.[illegible] g/l solution. The menthol was prior to each experiment diluted in water: 0.01 M quinine mono-hydrochloride (QHCl), 0.05-M acetic (HAc), 0.5-M sodium chloride (NaCl), 1 M sucrose (sucrose) and 2.5-M ethyl alcohol (alcohol). The same set of stimuli without menthol was also used. In one series of experiment (0.03 M and 0.01 M HAc with 0.1 g menthol/l solution were used as gustatory stimuli.

Results

The response to plain menthol solutions

Menthol solutions on the tongue elicited a slowly increasing discharge in the chorda tympani nerve as can be seen from the top recording of Fig. 1. The lowest concentration used, 0.05 g menthol/l water, gave a hardly perceptible increase of the recorded neural activity. The response to 0.1 g menthol/l was more evident, though the onset of discharge was slow and had a latency of a few sec. However, it gave an unquestionable increase of the neural activity. This activity seemed to reach a steady state after about 1/2 min, if the flow of menthol was sustained. The response to 0.2 g menthol/l water was stronger. It reached a maximum after about 1/2 min after which the neural activity declined. The general picture of the chorda tympani response to stimulation with menthol is similar to that of alcohol (ethanol). This can be seen by comparing the top and bottom recordings in Fig. 1. The main difference was observed when stimulation was interrupted and the stimulus rinsed away with water. Then the recording showed little of the strong burst of activity which can be seen after alcohol.

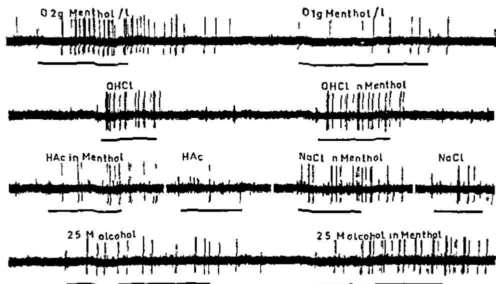


Fig 2 shows the response of a fine branch of the chorda tympani nerve to a series of gustatory stimuli The nerve impulses were recorded with a Statos I recorder Time marker 1/10 sec and 1/sec

The effect of plain menthol was also studied in a number of single fibre preparations Fig 2 is an example of such a recording In the top recording it can be seen that menthol itself elicited a slowly increasing spike activity without any initial burst of activity This pattern was repeated in all other fibres observed With regard to the relation between spike activity and menthol concentration it was observed that the response to 0 05 g menthol/l often was not perceptible, while the activity caused by the strongest solution reached a peak and then very often decreased if the stimulation was extended The top recordings in Fig 2 demonstrate the response to the two stronger concentrations used in the series of experiment

In Fig 3 the magnitude of the summated responses as well as the maximum impulse frequency for some single fibres have been plotted versus the concentration of the menthol solutions used From the data obtained it can be suggested that there is a linear relation between the menthol concentrations used and the maximum responses elicited The figures within the brackets of Fig 3 show the number of observations for the summated data the circles while the figures without brackets display the number of observations for the single fibres the points

The relation between the response to menthol and that to cooling was also observed in the single fibres The temperature of the water flow over the tongue was lowered with a rate of about 1 5° C/sec from 33° C to about 16° C An increase of the impulse activity was then observed in most fibres though not in all No difference however was observed in the responses to menthol between the fibres that responded to cooling and those that did not

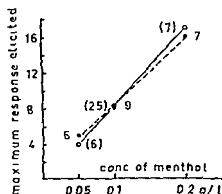


Fig 3

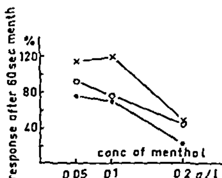


Fig 4

Fig 3 The diagram shows the relation between menthol concentration and response in the whole chorda tympani nerve, circles, and in a number of *single fibres, dots* *The figures along* the lines show the number of observations in each point

Fig 4 The diagram shows the relation between menthol concentration and magnitude of the summated response to the sapid solutions used after 60 sec of a menthol rinse The responses are all expressed in per cent of the responses to the same stimulus without menthol before the flow of menthol

The effect of adding menthol to some sapid solutions

In this part of the experiments 0 1 g menthol/l was chosen as being the most suitable concentration to add to the other solutions The weakest solution was regarded too weak, as it in itself elicited a very small response The strongest solution was apparently too strong, as it soon paralysed the chemoreceptors The responses to the sapid solutions with and without 0 1 g menthol/l were then recorded and compared It was then observed as can be seen in Fig 1 that adding menthol changed the gustatory response in two respects it slightly suppressed the initial burst of activity which normally appeared during the first seconds of stimulation with NaCl QHCl and HAc in the cat and it caused a slower decline or even an increase of the neural activity after the initial burst This can be seen by comparing the unbroken lines solution without menthol and the dotted lines, solution with menthol of each recording in Fig 1 Observations obtained in the single fibres gave similar results Their initial part of response was 25 %, S E 5 %, lowered when menthol was added to the sapid solutions But the later part of the activity, the sustained response was enhanced Fig 1 shows further that mentholic alcohol caused a faster increase of the neural activity than plain alcohol solution, while there was very little increase in activity when the mentholic alcohol was rinsed away after the stimulation Single fibre recordings gave similar results

It is possible that the observed enhancement of the sustained summated activity was the result of two kinds of fibres one responding to the stimulus in question while the other, as an effect of the menthol developed a slowly increasing activity

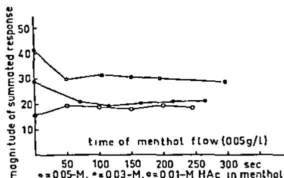


Fig 5 The responses to three concentrations of HAc have been plotted in the diagram versus the length of the preceding flow of 0 05 g menthol/l water Each symbol represents one observation

which added to the compound response thus giving the characteristics described above However, the single fibre recording of Fig 2 does not support this possibility It shows clearly that the same fibre responded with a somewhat different pattern when menthol was added to the solution This pattern is very similar to the pattern observed for the whole chorda tympani nerve Other recordings gave consistent observations

The effect of prolonged menthol exposure on the responses to other chemicals

In this part of the experiments the water rinse between the exposures was exchanged with a rinse of menthol The same concentrations of menthol as before were used The summated response to mentholic NaCl, QHCl, HAc, sucrose and alcohol solutions was recorded after different time periods of rinsing with menthol Stimuli containing menthol were used as otherwise the flow of menthol would have been interrupted giving time for the receptors to recover This would have affected the response to the next stimulation

It was then observed that the initial burst as well as the later part of the response to these solutions were affected The effect was related to the stimulus, the strength and the length of the preceding menthol rinse Fig 4 gives an idea of the relation between the response to the stimuli and the concentration of menthol The diagram consists of data from two cats The heights of the responses after one min of menthol rinse are expressed in per cent of the response to the same stimuli without menthol before the rinse Since the responses to NaCl 0 05-M HAc and QHCl showed small differences in the way they were affected by the extended rinse with menthol they were included in the same curve Fig 4 shows that the response to alcohol was most depressed It disappeared almost completely after one min exposure to the strongest menthol concentration On the contrary the response to sucrose was enhanced after one min rinsing with 0 05 and 0 1 g menthol/l But the strongest concentration of menthol depressed the response to sucrose after one min almost as much as it did with the others

For reasons which will be discussed later it was surmised that the strength of the response played an important role in these effects This assumption was tested for a

series of concentrations of HAc 0 05-M, 0 03 M and 0 01-M mixed with 0 1 g menthol/l solution Fig 5 shows the effect of a preceding rinse with 0 05 g menthol/l water on the responses of these three acid concentrations Fig 5 indicates that the response to the strongest acid concentration was most depressed while that of the weakest was somewhat enhanced The order of depressions were the same during rinsing with 0 1 g menthol/l solution Thus the response to 0 05 M HAc showed 73 % of its initial value, that to 0 03-M 79 % and that to 0 01-M 97 % after one min rinsing with 0 1 g menthol/l

The depression of the gustatory response, which occurred during menthol exposure, was reversible The neural response grew again when the menthol flow was exchanged with a water rinse The recovery was most evident for the response of alcohol stimulation A temporary enhancement of the responses was also observed during the recovery

Discussion

The present study shows that menthol in moderate concentration increases the neural response of the taste receptors to other sapid solutions, but also that it paralyses the receptors in higher concentration or during extended exposure These observations are in agreement with those described by other authors (Hensel and Zotterman 1951 Dodt *et al* 1952 Landgren *et al* 1953 Skouby and Zilstorff Pedersen 1954 1955)

It is possible to present a tentative explanation for the observations in this study *The studies by Kimura and Beidler (1961) and Tateda and Beidler (1964) demon*strate that gustatory stimulation depolarizes the taste cell Their studies show further that a stronger stimulus depolarizes more than a weaker but that the depolarization reaches a "limit value" beyond which it hardly can be pushed by stronger "normal" stimuli However "abnormal" stimuli like cocaine can push the receptor potential further or even reverse it It is also well known that during a continuous change of the receptor potential, the sensitivity of the receptor cell at first will increase, then decrease and finally disappear

It has been demonstrated earlier (Hellekant 1965) that the response to alcohol grows slowly and that the taste receptors continuously change their sensitivity to other stimuli during alcohol exposure Alcohol depolarizes the cell membrane (*cf* Dawson 1960) which has suggested that during alcohol exposure the response to another chemical is influenced by the level of depolarization at that moment

Menthol is more soluable in organic compounds than in water It may therefore accumulate in the structures of the taste cell The response to menthol as well as the effect of menthol on the excitability of the taste receptors are similar to those observed during alcohol exposure This indicates a similar effect by menthol as suggested before for alcohol That is the accumulation of menthol changes continuously the receptor potential and therefore also the excitability of the receptor cell

This change may also account for the differences between the effects of menthol on the responses of the other four stimuli used Stimulation with one of these four

shifts the receptor potential more rapidly than menthol does This shift adds to the slow change already caused by the menthol The response of the stimulus will be enhanced as long as the depolarization caused does not reach the limit value referred to earlier As soon as it does so a decrease of the neural response will be seen This decrease will be more pronounced with the diminishing potential space between the arbitrary limit value and the actual receptor potential Put in another way the response to weaker and weaker stimuli will be depressed with the shrinking potential space As for this study the response to sucrose is small in the cat both in single fibre and whole nerve recordings Its effect on the receptor potential is therefore according to Kimura and Beidler (1961) small It adds little to the change already caused by menthol QHCl stimulates more and therefore depolarizes more Consequently it will take longer time for the potential change caused by sucrose to come within the vicinity of the limit value of depolarization than it will for that of QHCl

This explanation can be supported or contradicted by studying the effect of menthol on the responses of a serie of different concentrations of the same stimulus Such a series will stimulate the receptors in the same way but to different extent From the hypothesis suggested above it can be concluded that the effect of menthol on their responses will show a different time course i e the response of the strongest solution will be depressed first or measured after equal time periods of menthol exposure most depressed

This consequence was repeatedly tested in one animal as described under results Acetic acid was chosen as stimulus because it elicits in moderate concentrations a good response in the chorda tympani nerve of the cat Further it has a fast rising receptor recovery function (Hellekant 1968) There are therefore few overlapping effects to be suspected between stimulations interspaced with reasonable intervals These series showed that the responses to the strongest acid are first and most depressed while those of the weakest are last and least depressed or even enhanced before they are depressed It is felt that these results support the idea of a changing receptor potential under the influence of menthol as the cause for the effects observed

No relation was observed between the responses of the taste fibres to cooling and to menthol as described before The observation may be of interest since menthol is regarded as a substance that gives a cool taste Hensel and Zotterman (1951) studied the cooling effect of menthol on the thermoreceptors of the tongue They found that the increase of the response which they observed when menthol was applied to the tongue during cooling was caused by an increased excitability of the receptors due to the menthol present but not by any measurable physical cooling by menthol There is therefore no reason to expect a relation per se between the sensitivity to menthol and to cooling in the gustatory fibres which is in agreement with the results presented in this study

In summary it is hypothesized that the receptor cell depolarizes slowly during the exposure to menthol This depolarization gives a slowly growing neural response

which declines, when the receptor potential reaches a limit value or a steady state Stimulation with any of the stimuli representing the four taste qualities depolarizes more rapidly This depolarization adds to the one caused by menthol Their sum determines the response to the new stimulus which will be enhanced as long as the sum of the potential changes has not reached the 'limit value", otherwise it will be depressed

This study has been supported by grants from Svenska Maltdrycksforskningsinstitutet Swedish Medical Research Council grant No B 69 14X 2467 02 and Magnus Bergvalls Stiftelse

References

DAVSON H *A Textbook of General Physiology* J & A Churchill London 1960

DODT E A P SKOUBY and Y ZOTTERMAN The effect of cholinergic substances on the discharges from thermal receptors *Acta physiol scand* 1952 *28* 101—114

HELLEKANT G Electrophysiological investigation of the gustatory effect of ethyl alcohol II A single fibres analysis in the cat *Acta physiol scand* 1965 *64* 398—406

HELLEKANT G *Studies of the effect of ethyl alcohol on the receptors of the tongue* Thesis Stockholm 1965

HELLEKANT G Postexcitatory depression of gustatory receptors *Acta physiol scand* 1968 *74* 1—9

HENSEL H and Y ZOTTERMAN The effect of menthol on the thermoreceptors *Acta physiol scand* 1951 *24* 27—34

KIMURA K and L M BEIDLER Microelectrode study of taste receptors of rat and hamsters *J comp Physiol* 1961 *58* 131—139

LANDGREN S A P SKOUBY and Y ZOTTERMAN Sensitization of baroreceptors of the carotid sinus by acethylcholine *Acta physiol scand* 1953 *29* 381—388

SKOUBY A P and K ZILSTORFF PEDERSEN The influence of acetylcholine like substances menthol and strychnine on olfactory receptors in man *Acta physiol scand* 1954 *32* 252—258

SKOUBY A P and K ZILSTORFF PEDERSEN The influence of acetylcholine menthol and strychnine on taste receptors in man *Acta physiol scand* 1955 *34* 250—256

TATEDA H and L M BEIDLER The receptor potential of taste cell of the rat *J gen Physiol* 1964 *47* 479—486

Acta physiol. scand. 1969. 76. 369—375

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Isolation of Fibrinopeptides Formed during Intravascular Coagulation in Dogs

By

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Received 7 October 1968

Abstract

GRÖNDAHL, N J, S P MILLER and A C TEGER NILSSON *Isolation of fibrinopeptides formed during intravascular coagulation in dogs* Acta physiol scand 1969 *76* 369—375

Purified canine fibrinopeptide A was incubated in canine serum *in vitro* The trichloroacetic acid-soluble degradation products were isolated by preparative electrophoresis and identified by means of amino acid analyses Desargınino fibrinopeptide A and free arginine were found, indicating that C-terminal arginine is split off from fibrinopeptide A during incubation in serum *in vitro* An attempt was made to isolated fibrinopeptides and possible fibrinopeptide [illegible] electrophoretic mobility of the fractions and of their tryptic digests as well as the amino acid analyses indicated that one fraction consisted of fibrinopeptide A and the other of desarginino fibrinopeptide A

Several investigations made with purified fibrinogen *in vitro* have shown that thrombin induced fibrin formation is preceded by the release of two peptides, the fibrinopeptides, from the fibrinogen molecule (*cf* Blombäck 1967) It is therefore now generally assumed that thrombin induced fibrin formation *in vivo* is also related *to release of the fibrinopeptides from fibrinogen Until now, however, this has not* been proved and the fibrinopeptides have not been isolated from blood, clotted *in vivo*

The present investigation was an attempt to isolate the fibrinopeptides from canine blood after experimentally induced intravascular coagulation As fibrinopeptides may be degraded in blood (Teger Nilsson and Blombäck 1967, Teger-Nilsson 1969), the products formed during incubation of purified canine fibrinopeptide A in serum *in vitro* were first identified

Material and methods

Animals Mongrel dogs, weighing 15—20 kg, were used for the isolation experiments

[illegible]

1954), and chlorination reactions (Reindel and Hoppe 1954)

Amino acid analyses were made in an Auto-Analyzer (Technicon Instruments Corp, Chauncey, N Y) Prior to chromatography, the samples were hydrolyzed in 5 7 M hydrochloric acid, at 110° C, under N_2 for 22 hrs

Peptide separation was made on an automatic peptide analyzer (Technicon Instruments Corp, Chauncey, N Y)

Experiments and results

In vitro degradation of fibrinopeptide A in serum

1 5 mg of fibrinopeptide A was dissolved in 150 μl of serum and 1 μl of toluene, and incubated for 24 hrs at 37° The same volume of serum alone was used as blank After incubation, proteins were precipitated at 0° C in 10 % trichloroacetic acid (TCA) The precipitate was discarded, and TCA was removed from the supernatant by extraction with ether The samples were lyophilized, and the residues were submitted to high voltage electrophoresis on paper Guide strips from each side of the paper were cut out, and the bands were identified with the ninhydrin, Sakaguchi and chlorination reactions The bands on the remaining paper sheet were eluted with 3—4 ml of distilled water The eluates were lyophilized, and the residues were analyzed for their amino acid composition after hydrolysis

The electrophoretic picture of the incubate, as seen on the guide strips, is shown in Fig 1, where the electrophoretic mobilities of unchanged fibrinopeptide A and the serum blank are also given During incubation of fibrinopeptide A in serum, three fractions appeared Fraction II was located at the same place where the serum blank showed a band and may therefore have consisted of serum components Frac-

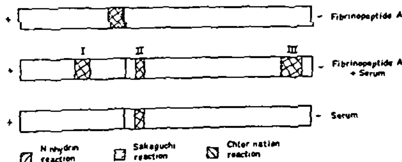


Fig 1 Electrophoretic picture of fibrinopeptide A after 24 hrs digestion in serum The electrophoresis was run on paper for 2 hrs in 0 1 M pyridine acetate buffer, pH 5 5, at 45—55 V/cm, 20—100 mA.

tions I and III had apparently been formed from the fibrinopeptide during incubation. Fibrinopeptide A thus seemed to be degraded during incubation in serum

The results of the amino acid analysis are shown in Table 1 Fraction I had the same amino acid composition as canine fibrinopeptide A (Blombäck *et al* 1964), with the exception of a low arginine content Fraction I thus seemed to consist of fibrinopeptide A without its C terminal arginine Fraction II contained several amino acids None of them was comparatively more abundant than in the serum blank, and they could therefore all have been derived from serum Fraction III contained mainly arginine, and no arginine was found in the blank It thus seems that the arginine is split off from the C terminal end of the fibrinopeptide A No splitting from the N terminal end, and no other degradation of importance seemed to have occurred

The blood was immediately centrifuged at 2000×*g* for 30 min at 0° and "plasma was

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The residue (2 7 g) was then dissolved in 9 ml of 1 6 M acetic acid and applied to a column of Sephadex G10 16 cm²×56 cm equilibrated with 1 6 M acetic acid The column

fraction G10—1 on paper (45
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(*cf* Iwanaga
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on before and
after alkaline hydrolysis The chromatogram is shown in Fig 2 The fractions were pooled as

derivatives are expected were submitted to further separation by preparative electrophoresis 20 µl of the fractions were applied to a thin layer plate along a 17-cm line situated 12 cm from the anode Electrophoresis was run for 2 hrs under the conditions described above After the run the location of the bands was visualized by staining two guide strips (5 mm) with ninhydrin The cellulose at the remaining unstained bands was collected on a sintered glass filter by suction and subsequently eluted with 4 ml of 1 6 M acetic acid The eluates were lyophilized and submitted to amino acid analyses after hydrolysis

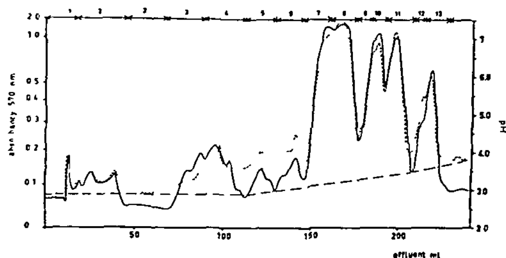


Fig 2 Chromatogram of the peptide separation of fraction G 10 2 The effluent volume given in the figure is the volume after 27 % had been withdrawn for analyses
——— ninhydrin reaction without hydrolysis
– – – – – ninhydrin reaction after alkaline hydrolysis
— · — · — pH

TABLE I Amino acid composition of the electrophoretic fractions obtained after incubation of canine fibrinopeptide A in canine serum *in vitro* Structure of canine fibrinopeptide A (*cf* Blombäck 1964) NH_2 Thr Asn Ser Lys Glu Gly Glu Phe Ilu Ala Glu Gly Gly Gly Val Arg OH

Amino acid	Fraction I		Fraction II		Fraction III	
	μmoles	residues/mole	sample μmoles	blank μmoles	sample μmoles	blank μmoles
Asp	0 030	1 0 (1)	0 006	0 004	0 003	trace
Thr	0 025	0 8 (1)	0 013	0 014	—	—
Ser	0 018	0 7 (1)	0 009	0 011	—	trace
Glu	0 088	2 9 (3)	0 018	0 027	0 002	trace
Pro	—	—	trace	0 014	—	—
Gly	0 130	4 2 (4)	0 019	0 022	0 004	0 004
Ala	0 031	1 0 (1)	0 015	0 027	—	—
Val	0 030	1 0 (1)	0 007	0 010	—	—
Ilu	0 031	1 0 (1)	0 004	0 005	—	—
Leu	—	—	0 004	0 003	—	—
Tyr	—	—	—	trace	—	—
Phe	0 029	0 9 (1)	0 002	0 004	—	—
Lys	0 033	1 1 (1)	—	0 008	—	—
Arg	0 004	0 1 (0)	—	—	0 058	—

TABLE II Amino acid composition of two of the fractions obtained from the blood of thrombin treated dogs

Amino acid	Fraction Au 1 3			Fraction Au-4-1		
	µmoles	residues/mole		µmoles	residues/mole	
Asp	0 039	1 4	(1)	0 031	1 6	(1—2)
Thr	0 025	0 9	(1)	0 017	0 9	(1)
Ser	0 030	1 1	(1)	0 022	1 1	(1)
Glu	0 097	3 5	(3 -4)	0 066	3 4	(3)
Pro	—	—	(0)	—	—	(0)
Gly	0 138	5 0	(4—5)	0 095	4 8	(4—5)
Ala	0 033	1 2	(1)	0 029	1 4	(1)
Val	0 030	1 0	(1)	0 020	1 0	(1)
Cys/2	0 010	0 4	(0)	—	—	(0)
Met	—	—	(0)	—	—	(0)
Ilu	0 026	0 9	(1)	0 018	0 9	(1)
Leu	0 005	0 2	(0)	trace	—	(0)
Tyr	trace	—	(0)	trace	—	(0)
Phe	0 022	0 8	(1)	0 020	1 0	(1)
Try	—	—	(0)	—	—	(0)
Orn	trace	—	(0)	trace	—	(0)
Lys	0 027	1 0	(1)	0 020	1 0	(1)
His	0 007	0 3	(0)	trace	—	(0)
Arg	trace	—	(0)	0 015	0 8	(1)

Only two of the bands denoted as fractions Au 1 3 and Au-4 1 had an amino acid composition which could be related to the fibrinopeptides (Table II) The amino acid composition of fraction Au 1 3 was in resonable agreement with that of *canine desarginino-fibrinopeptide A Small amounts of cysteine leucine and histidine* were however present indicating that the peptide was not completely pure The electrophoretic mobility of fraction Au 1 3 shows that it was somewhat more acidic than peptide A (Fig 3)

The amino acid composition of fraction Au-4 1 was the same as that of unchanged fibrinopeptide A apart from slightly large amounts of aspartic acid glutamic acid glycine and alanine It is therefore likely that fraction Au-4 1 consisted of unchanged fibrinopeptide A although not completely pure This view is strengthened by the finding that fraction Au 4 1 had the same electrophoretic mobility as purified fibrinopeptide A (Fig 3)

None of the other analyzed acidic fractions could be related to the fibrinopeptides Neither fibrinopeptide B nor derivatives of fibrinopeptide B were found In this connexion it is perhaps worth mentioning that one of the fractions Au 5 1, contained mainly glutamic acid glycine and cysteine in roughly equimolar amounts, and may therefore be identical with glutathione

To obtain further evidence that fractions Au-4 1 and Au 1 3 consisted of fibrino-

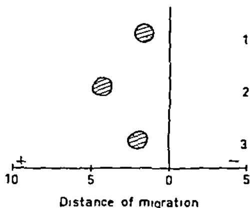


Fig 3 Thin layer electrophoresis in 0 1 M *pyridine acetate buffer, pH 5 5, for 2 hrs at* 15 V/cm 15 mA Identification was made with the ninhydrin and Sakaguchi reactions 1 Fibrinopeptide A 2 Fraction Au 1-3 3 Fraction Au 4-1

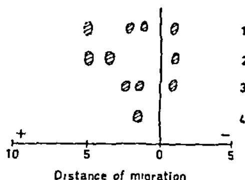


Fig 4 Thin layer electrophoresis in 0 1 M pyridine acetate buffer, pH 5 5, *for 1 5 hr* at 15 V/cm, 15 mA Identification with the ninhydrin reaction
1 Tryptic digest of fraction Au 4 1 2 Tryptic digest of fraction Au 1-3 3 Tryptic digest of fibrinopeptide A 4 Fibrinopeptide A

peptide A and its degradation product, these fractions and purified fibrinopeptide A were submitted to tryptic digestion, and the electrophoretic mobility of the digests compared The fractions were each dissolved in 50 μl of 0 2 M ammonium bicarbonate pH 8 6 Trypsin (2 5 μg in 0 5 μl of 0 001 M hydrochloric acid) was added The samples were digested for 4 hrs at 37° C and then lyophilized The residues were dissolved in 5 μl of distilled water and submitted to thin layer electrophoresis The results are shown in Fig 4 Two new fractions are expected after tryptic digestion of fibrinopeptide A, since it contains a lysyl bond but no other trypsin-susceptible bonds The tryptic digest of the reference peptide showed three spots, the most acidic one representing the C-terminal part of the molecule, the next unchanged peptide, and the basic spot the N-terminal part of the molecule The tryptic digest of fraction Au-4-1 gave the same main spots as did the digest of fibrinopeptide A The digest of fraction Au 1-3 showed two acidic spots, probably representing the C-terminal part and the unchanged peptide, and a basic spot at the same place as the reference The results strengthen the view that fractions Au 4-1 and Au-1-3 are fibrinopeptide A and desarginino-fibrinopeptide A

Discussion

In vitro incubation of canine fibrinopeptide A in canine serum showed that the C-terminal arginine was split off, possibly by carboxypeptidases in serum This is the same finding as in similar experiments on human material (Teger-Nilsson 1969) In contrast to the findings for human fibrinopeptide A, there was no detectable degradation from the N-terminal end of the canine fibrinopeptide

Two peptides resembling fibrinopeptides were isolated from the blood of thrombin-treated dogs The behaviour during the isolation procedure, the electrophoretic

mobility of the peptides and of their tryptic digests, and the amino acid composition indicated that these peptides were fibrinopeptide A, and desarginino-fibrinopeptide A

The yield of the peptides was very low There are, however, several reasons why a high yield is improbable In view of the size of the fibrinopeptides, it is possible that they are fairly rapidly excreted in the urine More extensive degradation of fibrinopeptide A might also have occurred *in vivo* than *in vitro* Finally, the yield of the peptides in the purification procedure is probably fairly low

Neither fibrinopeptide B nor derivatives of this peptide were found in the blood after thrombin treatment In several species fibrinopeptide B is split off from fibrinogen more slowly than fibrinopeptide A, and fibrin can be formed without release of fibrinopeptide B (*cf* Blomback 1967) The absence of fibrinopeptide B does not however, necessarily imply that it is not splitt off from fibrinogen during intravascular coagulation Extensive degradation of the peptide *in vivo* might have occurred moreover, it might have been excreted in the urine or lost in the separation procedure

In conclusion it can be stated that C terminal arginine is split off from canine fibrinopeptide A during incubation in canine serum *in vitro* Not only fibrinopeptide A but also desarginino-fibrinopeptide A are therefore expected to circulate in the blood during states of intravascular coagulation In an attempt to isolate the fibrinopeptides from the blood of thrombin treated dogs two fractions were identified, which could be correlated to the fibrinopeptides One of the fractions was mainly fibrinopeptide A and the other desarginino fibrinopeptide A

This investigation was supported by grants from Karolinska Institutet Swedish Medical Research Council No K68 19X 520-04 and National Institutes of Health No HE 07379 04 to Laborator *Birger Blomback*

We wish to thank Laborator Birger Blomback for much support and valuable discussions

References

BLOMBACK B *Fibrinogen to fibrin transformation Blood clotting enzymology* Ed W H Seegers Academic Press N Y and London 1967 pp 143—215

BLOMBACK B M BLOMBACK P EDMAN and B HESSEL Human fibrinopeptides Isolation characterization and structure *Biochim biophys Acta* (Amst) 1966 *115* 371—396

BLOMBACK B R F DOOLITTLE and M BLOMBACK Fibrinogen structure and evolution *Protides of the biological fluids* Ed H Peeters Elsevier publishing Co Amsterdam 1964 pp 87—94

IWANAGA S P WALLEN N J GRONDAHL A HENSCHEN and B BLOMBACK On the primary structure of human fibrinogen II Isolation and characterization of N terminal fragments from plasmic digest *Europ Biochem J* In press

LEVY A L and D CHUNG Two-dimensional chromatography of amino acids on buffered papers *Anal Chem* 1953 *25* 396—399

REINDEL F and W HOPPE Über eine Farbemethode zum Anfärben von Aminosauren Peptiden und Proteinen auf Papierchromatogrammen und Papierelektropherogrammen *Chem Ber* 1954 *87* 1103—1107

ROCHE J N V THOAI and J L HATT Métabolisme des derivés guanidyles III Analyse chromatographique des derivés guanidylés *Biochim biophys Acta* (Amst) 1954 *14* 71—75

TEGER NILSSON A C Degradation of human fibrinopeptides A and B in blood serum *in vitro* *Acta chem scand* In press

TEGER NILSSON A C and B BLOMBACK Degradation of human fibrinopeptides in serum *Acta chem scand* 1967 *21* 307—309

Acta physiol. scand 1969. 76. 376—382

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Non-Uniform Blood Flow in the Left Ventricular Wall of Dogs Measured by the Xe-133 Wash-out Technique

By

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Received 15 October 1968

Abstract

ANDERSEN, H H BAGGER and H. GOTZSCHE *Non-uniform blood flow in the left ventricular wall of dogs measured by the Xe-133 wash-out technique* Acta physiol scand 1969 *76* 376—382

Intra myocardial injections of Xe 133 into the left ventricular wall revealed time activity curves which indicate that blood flow in the subepicardial layers is 30 % greater than in the subendocardial layers A two-compartment model of the ventricular wall was therefore considered, and a good correlation was found between this model and the results of graphical and computer analyses of time activity curves obtained by injections of Xe 133 into the left coronary artery The flows of these two compartments and flows after superficial and deep intra myocardial injections were not identical Thus the myocardial two-compartment model must be abandoned. A gradual decrease of flow from the subepicardial to the subendocardial surface is suggested. If this is true then the wash-out curve after intra-coronary injection of Xe 133 is multi-exponential and thereby inaccessible to graphical analysis

It is commonly accepted that coronary blood flow is uniform throughout the left ventricular wall (Johnson, Cavert and Lifson 1952 Herd, Hollenberg and Thorburn 1962) Sullivan *et al* (1967) found that Kr-85 clearance constants measured after intra myocardial deposition of tracer at a uniform depth of 3 mm did not differ from those obtained after injection of tracer into the left anterior descending artery However, estimation of local myocardial blood flow from the disappearance rate of intra myocardial deposits of NaI-131 (Kirk and Honig 1964b) showed that tissue blood flow in the deep layers of the left ventricular wall was slower than in the superficial layers

In the present study local myocardial blood flow was estimated from the disappearance rate of the radioactive inert gas Xe 133 deposited at two different depths of the free left ventricular wall In addition Xe-133 was injected as a bolus into the left coronary artery, to see if a possible inhomogeneity of flow could be demonstrated as a non-mono-exponential clearance curve and to compare the resultant flows with flows obtained by intra myocardial injections of Xe 133

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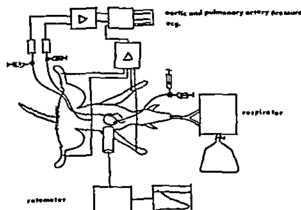


Fig. 1. Experimental plan

Methods

The experiments were performed on mongrel dogs of 15—35 kg. Thiomebumal-sodium (Leopental®) was given i.v. (15—20 mg per kg), and respiration was maintained with atmospheric air through an endotracheal tube using an Engstrom ventilator (Fig. 1).

Catheters were introduced through the right femoral artery and vein into the aorta and pulmonary artery to allow continuous observation of pressures together with simultaneous recording of the ECG. Repeated measurements of oxygen and carbon dioxide tension, oxygen saturation and pH in the arterial blood were performed. Cardiac output was measured by the direct Fick method.

The right carotid artery was exposed and a Sones no. 8 coronary catheter was inserted, *the tip was placed just within the left coronary artery under fluoroscopy and its position* confirmed by injection of radiopaque material. Thoracotomy was performed through a left lateral approach and the pericardium was opened. Xe 133 was then injected either directly into the myocardium of the left ventricle or into the left coronary artery.

Xe 133 was obtained from The Radiochemical Centre, Amersham, England, in batches of 10—12 mC in 10 ml saline. Volumes of 0.1 ml were used for intra myocardial injections and 0.3—0.5 ml for injections into the coronary artery, giving peak rates of about 1000—2000 *counts per sec. Needles with an external diameter of 0.4 mm were used for intra-myocardial* injections, and the deposits were applied in depths of 2 mm for superficial and 8 mm for deep flow measurements. The sites of intra myocardial injections are shown in Fig. 2. Immediately following intra coronary injections the catheter was flushed with isotonic glucose to remove the xenon.

Myocardial radioactivity was detected with a 1 ¾ inch thallium activated sodium iodide crystal. A wide angle lead collimator allowed detection of activity from the whole heart. *Wash-out was recorded by means of a rate meter operating with a 1 or 4 sec time constant* and a strip chart linear/logarithmic recorder with a paper speed of 3.8 cm per min.

Calculations

Assuming equal perfusion of all regions of the myocardium containing the radioactive inert gas, i.e. a one-compartment model, the wash-out after local injection of the tracer can be expressed by (Kety, 1949)

$$Q_t = Q_0 e^{-kt} \quad (1)$$

where Q_t and Q_0 are the count rates of myocardial radioactivity at time t and zero, and the rate constant

$$k = \ln 2/T_{1/2} = F/(V \cdot \lambda) = (F \cdot \rho)/(W \cdot \lambda)$$

where $T_{1/2}$ is the biological half life of the tracer, λ the tissue-blood partition coefficient, W and ρ the weight and specific gravity of the myocardium and F the blood flow through W grams of tissue. Assuming a λ of 0.77 and a ρ of 1.00, flow can be calculated from

$$F = (W \cdot \lambda \cdot \ln 2)/(\rho \cdot T_{1/2}) = (100 \cdot 0.77 \cdot 0.693)/(1.00 \cdot T_{1/2}) = 53/T_{1/2} \quad (2)$$

and is expressed as ml/100/g/min.

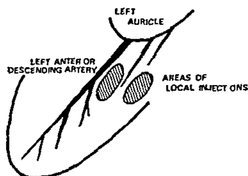


Fig 2

Fig 2 Sites of intra myocardial injections of Xe 133

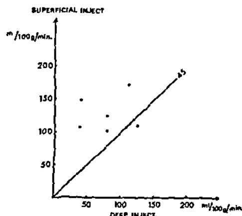


Fig 3

Fig 3 Flows obtained after superficial intra myocardial injections compared with flows after deep, intra myocardial injections of Xe 133

Clearance curves after local injection of the tracer are seldom mono-exponential they show usually a decrease in the relative slope with time However, if diffusion equilibrium is assumed to exist initially blood flow can be calculated from the inital slope of the curve (Lassen 1967) Our flows after intra myocardial injections have been calculated using the initial slope of the clearance curves and equation (2)

Assuming n independently perfused compartments in parallel in the myocardium with diffusion equilibrium in each compartment and no exchange of tracer between them the wash out of the gas can be expressed by

$$Q_t = Q_{1(0)} e^{-k_1 t} + Q_{2(0)} e^{-k_2 t} \quad + Q_{n(0)} e^{-k_n t} \tag{3}$$

where $Q_{1(0)}$ $Q_{2(0)}$ are the activities of compartment 1 2 at time zero k_1 k_2 the rate constants of each compartment

Though a semi logarithmic plot of equation (3) does not reveal a straight line the individual terms can be obtained by graphical resolution of the curve A standard resolution procedure has been used for clearance curves obtained after intra arterial injections of Xe 133 (see *e g* Haggendal Nilsson and Norback 1965)

In addition to graphical resolution compartmental analysis of clearance curves recorded after intra coronary injections of Xe 133 has been performed by an electronic computer A computer programme was constructed by The Computing Centre (University of Århus) which after subtraction of wash-out from the final compartment gave a least square best fit by successive approximations of two exponential functions A plotter operated by the computer drew for visual comparison the experimental curve minus the final compartment and the sumcurve of the exponentials found (Fig 6)

Results

Wash-out curves of Xe 133 showed a final, slow removal of radioactivity after both intra myocardial and intra-coronary injections (Table I final compartment) An equally slow wash-out was found by simultaneous recording over the left femur of the dogs, and was of the same order of magnitude as flows of the third compartment found by xenon clearance of the brain (Haggendal *et al* 1965) and of the kidney (Ladefoged 1966) Thus this final compartment has been considered to be due to removal of Xe 133 from myocardial fat scatter from extra myocardial tissue and to

TABLE I

Site of injection	Number of observations	Compart 1 Mean ± S D ml/100 g/min	Compart 2 Mean ± S D ml/100 g/min	Final compart Mean ± S D ml/100 g/min
Left coronary artery	17	145 ± 30	49 ± 11	4 ± 1
Superficial layer of myocardium	14	108 ± 26		3 ± 1
Deep layer of myocardium	14	71 ± 24		2 ± 1

Table I Mean flow values after intra-coronary and intra myocardial injections of Xe 133

recirculation and it has been subtracted from the original time activity curve after both intra myocardial and intra coronary injections of Xe-133

A comparison between flows calculated from the initial slope of the wash out curves obtained from 14 pairs of superficial and deep intra myocardial injections into the same area is shown in Fig 3 The difference of 30 % between superficial and deep flows is significant with regard to mean values (Table I) and when estimated from paired observations ($p < 0.005$) To decide whether the fast removal of xenon after superficial injections could be due to an escape of xenon through the epicardial surface Xe-133 was injected into the superficial layers of the myocardium and activity recorded before during and after constriction of the left coronary artery (Fig 4) The slight decrease in radioactivity during constriction is too small to account for the difference between superficial and deep flows and has been ascribed to collateral coronary blood flow (Johansson Linder and Seeman 1964)

Semi logarithmic clearance curves of myocardial radioactivity recorded for 20 min after Xe 133 bolus injection into the left coronary artery were submitted to graphical compartmental analysis Of 18 injections 17 revealed a sum of two ex-

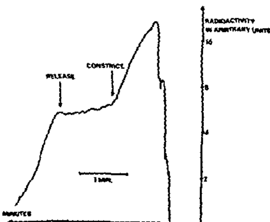


Fig 4 Time activity curve recorded after superficial intra myocardial injection of Xe 133 showing the effect of constriction of the coronary artery

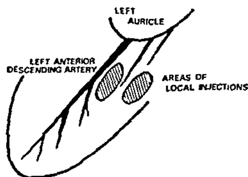


Fig 2

Fig 2 Sites of intra myocardial injections of Xe 133

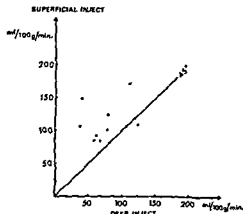


Fig 3

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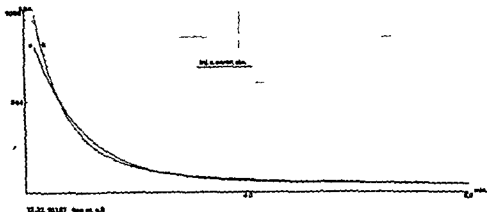


Fig 6 A representative result after injection of Xe 133 into the left coronary artery The dotted line is the recorded time activity curve *minus* wash-out from the final compartment Curve a) shows the best fitting mono-exponential function and curve b) the best fitting sum curve of two approximated exponentials.
Ordinate Counts per sec Abscissa Time in minutes

The demonstration by Gillespie and Love (1967) of a myocardial uptake of intravenously injected Rb-86 which is greater in the deep layers than in the superficial layers seems inconsistent with our results According to Moir (1966) myocardial uptake of Rb-86 depends on the time the isotope remains in the capillary bed A greater subendocardial than subepicardial blood content (Myers and Honig 1964) indicates that capillary density is greater in the deep layers than in the superficial layers In view of these findings the Rb-86 measurements are not necessarily inconsistent with our results

According to Johansson *et al* (1964) and Linder (1966) the myocardial clearance after intracoronary injection of saline Kr 85 and Xe 133 follows a mono-exponential function With an extended period of recording this could not be demonstrated in our experiments Subjected to graphical resolution they revealed a sum of two exponentials and analysed by computer they showed a good fit to a sum of two exponential functions

The finding of non-mono-exponentialities with a good fit to a sum of two exponential functions does not demonstrate that two main flow levels exist in the left ventricular wall A two compartment model is compatible with the anatomical distribution of myocardial blood vessels as it is possible to differentiate between separate subepicardial and subendocardial artery supplies (Estes *et al* 1966) However if one accept that local tissue flow in the different depths of the wall is mainly influenced by the intramural pressure there may actually exist a gradual decrease of local flow from the subepicardial to the subendocardial surface Hence the wash out curve after intra-coronary injection of xenon is multi exponential and inacces-

sible to graphical analysis. This invalidates a myocardial two-compartment model, but might explain the lack of agreement in comparing the flow of compartment 1 and 2 after intra-coronary injection with flows after the intra myocardial injections

This work has been supported by Danish State Research Foundation, L 48/66.

We are indebted to C. Hilberg, The Computing Centre, University of Aarhus, for the computer analyses.

References

Estes Jr., E. H., M. L. Entman, H. B. Dixon and D. B. Hackel, The vascular supply of the left ventricular wall. *Amer Heart J* 1966 *71* 58—67

Gillespie, W. J. and W. D. Love, Gradients in the regional rates of myocardial Rubidium-86 clearance in tranquilized dogs. *Circulat Res* 1967 *20* 606—615

Haggendal, E., N. J. Nilsson and B. Norback, On the components of Kr-85 clearance curves from the brain of the dog. *Acta physiol. scand* 1965 *66* Suppl. 258 5—25

Herd, J. A., M. Hollenberg and G. D. Thorburn, Myocardial blood flow determined with Krypton-85 in unanesthetized dogs. *Amer J Physiol.* 1962 *203* 122—124

Johansson, B., E. Linder and T. Seeman, Collateral blood flow in the myocardium of dogs measured with Krypton 85. *Acta physiol scand* 1964 *62* 263—270

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Johnson, J. R. and J. R. Di Palma, Intramyocardial pressure and its relation to aortic blood pressure. *Amer J Physiol* 1939 *125* 234—243

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Kirk, E. S. and C. R. Honig, Nonuniform distribution of blood flow and gradients of oxygen tension within the heart. *Amer J Physiol.* 1964b *207* 661—668.

Ladefoged, J., Measurement of the renal blood flow in man with the [133]Xenon wash-out technique. *Scand J clin Lab Invest* 1966 *18* 299—315

Lassen, N. A., On the theory of the local clearance method for measurement of blood flow including a discussion of its application to various tissues. *Acta med scand* 1967 Suppl. 472 136—143

Laszt, L. and A. Muller, Der myokardiale Druck. *Helv physiol. pharmacol. Acta* 1957 *15* 82—106

Linder, E., Measurements of normal and collateral coronary blood flow by close arterial and intramyocardial injection of Krypton[85] and Xenon[133]. *Acta physiol. scand* 1966 *68* Suppl. 272 5—31

Moir, Th. W., Measurement of coronary blood flow in dogs with normal and abnormal myocardial oxygenation and function. *Circulat Res* 1966 *19* 695—699

Myers, W. W. and C. R. Honig, Number and distribution of capillaries as determinants of myocardial oxygen tension. *Amer J Physiol* 1964 *207* 653—660

Sullivan, J. M., W. J. Taylor, W. C. Elliott and R. Gorlin, Regional myocardial blood flow. *J clin Invest* 1967 *46* 1402—1412.

Acta physiol. scand 1969 76. 383—384

From the Department of Physiology, Karolinska Institutet Stockholm, Sweden

Antagonism between Prostaglandin E_2 and Phenoxybenzamine on Noradrenaline Release from the Cat Spleen

By

P Hedqvist

Nerve stimulation of the spleen has been shown to cause increased overflow of noradrenaline (NA) (Brown and Gillespie 1957), and of prostaglandin (PG), mainly as PGE_2 (Davies, Horton and Withrington 1967) Recently infusion of PGE was found to reduce the outflow of NA from the spleen in response to nerve stimulation, suggesting that PG might exert a braking action on the process of NA release (Hedqvist 1969)

Since the *a* receptor blocking drug phenoxybenzamine (PBA), which has been shown to increase the outflow of NA from the spleen in response to nerve stimulation (Brown and Gillespie 1957), inhibits the release of PG from that same organ (Davies *et al* 1967), the present experiments were carried out to study the possibility of antagonism between PBA and PGE_2 on the release of NA from the spleen induced by nerve stimulation

Cat spleens were isolated and perfused at a constant rate of 10 ml/min with a modified Krebs Henseleit s solution as described elsewhere (Hedqvist and Stjarne 1969) The effluent from the spleen was collected in 10 ml fractions and analyzed fluorimetrically for NA The splenic nerves were stimulated for 20 sec at 10/sec before and in the presence of PGE_2 (0 6 μg/ml) and/or PBA (1 0 μg/ml)

Nerve stimulation led to a considerable increase in the outflow of NA from the spleen Addition of PBA to the perfusion medium resulted in a several fold increase of this response Simultaneous infusion of PGE strongly counteracted this effect of PBA leading to NA output figures close to those from the preceding control stimulation (Fig 1) After cessation of the infusion of PGE_2 (PBA still in the medium) the NA overflow response to nerve stimulation gradually increased only to be markedly reduced often to a level close to that caused by control stimulation, on renewed infusion of PGE Thus PGE_2 was found to inhibit the onset of the PBA induced potentiation of NA outflow in response to nerve stimulation, and also to reduce the effect of PBA on the NA outflow once it was established

The present study supports the previously presented concept that PGE_2 may exert a braking action on the release of NA from the nerves (Hedqvist 1969) The results also demonstrate the existence of antagonism between PGE_2 and PBA on transmitter release from the splenic nerves It is thus conceivable that part of the increase of

the [illegible] capacity of the salt gland. The purpose of the present investigation is, [illegible] give an account of the flow rates and electrolytic concentrations in the [illegible] stimulated salt gland as measured by a new experimental device and to [illegible] the data with those published earlier.

Material

[illegible] birds (1—2 years of age, body weight 780—1000 g) of *Larus argentatus* were trapped and kept in captivity from 18 hours to one week. They were fed wastes of fish, and bread as [illegible] seawater *ad libitum*. Totally 15 birds were used.

Methods

[illegible] An initial dose of 1.0 ml/kg b.w. of Mebumal sodium (6 %) in the femoral muscle [illegible] deep anesthesia after 30 min at most. This was maintained for 8—10 hrs by 0.1— [illegible] min.

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[illegible] Parasympathetic secretory nerve fibres in [illegible] *palat*[illegible] of the facial [illegible] with the [illegible] *ophthalmicus* of the trigeminal nerve 5—7 mm in the anterior upper [illegible] Numerous synapses occur along the common nerve and this ganglionic mass [illegible] *ganglion ethmoidale* (Cords 1904) or *ganglion sphenopalatinum* (Webb 1957). In [illegible] the position of the stimulation electrodes to these synapses the nerve was cut in [illegible] stained with hematoxylin and eosin and the number of ganglionic cells calculated as [illegible] 1.

[illegible] was already 2—3 mm before it joins the [illegible] *palatinus*. Along the common [illegible] two ganglionic masses are especially noticeable, the first one at the common trunk [illegible] other at a point where this divides into fibres to the salt gland and to the Harderian gland. However, it is histologically impossible to discriminate between the secretory fibres of the two glands. Nevertheless it is evident that the salt gland is provided with fibres from the distal ganglionic mass.

The position of the stimulating cathode at x in Fig. 1 indicates that the stimulation is at least partly presynaptic.

Stimulator. A Grass Stimulator S9 was used. The electrodes were of stainless steel. If not otherwise stated the duration was 1 msec, intensities 1—5 V, and frequencies 20—30 sec. Stimulation time was 5—6 min.

The cut ends of the opthalmic and palatine rami were tied together and pulled into a plastic tube in order to isolate them, and stimulation needle electrodes penetrated into the plastic tube lumen, the cathode being at the distal part of the nerve.

Flow measuring. For continuous recording of the secretion it was necessary to construct a new type of flowmeter capable of recording very slow flow rates. This flowmeter made use of the light-conducting properties of perspex material and was constructed in the following way. Two circular perspex discs were placed as shown in Fig. 2A, the one light-shielded from the other with the exception of a small groove in the outer disc into which a polyethylene tubing (Intramedic PE 200 1.9 mm o.d., 1.4 mm i.d.) was placed.

The inner disc transmitted the light from a bulb in its center through the transparent plastic tube in the groove of the outer disc. Then the light followed the perspex material to the center of the outer disc where a phototube (ORP 60) was placed on a plastic knob in contact with the disc. The polyethylene tubing outside the flowmeter contained an ink column. The end of this tubing was connected to the catheter from the gland duct. When flow occurred from the gland the ink column was pushed into the light pathway of the flowmeter thus diminishing the current through the phototube.

An inkwriter (VARIAN G-11A) recorded the voltage tapped over a resistance in the phototube circuit (Fig. 2A).

The system showed linearity from 0—450 mm³.

In some cases the salt concentration of the secretion was measured when the flow rate was known. This required clean sampling devices and contamination with the ink column of the flowmeter had to be avoided. Introducing an air filled volume between the duct catheter and the flowmeter gave an error in the flow rate measuring which could be compensated by measuring the compression of the above mentioned air bubble (Fig. 2B). The compression

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References

ESTES JR E H M L ENTMAN H B DIXON and D B HACKEL, The vascular supply of the left ventricular wall *Amer Heart J* 1966 *71* 58—67
GILLESPIE W J and W D LOVE Gradients in the regional rates of myocardial Rubidium 86 clearance in tranquilized dogs *Circulat Res* 1967 *20* 606—615
HAGGENDAL E N J NILSSON and B NORBACK On the components of Kr-85 clearance curves from the brain of the dog *Acta physiol scand* 1965 *66* Suppl 258 5—25
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LADEFOGED J Measurement of the renal blood flow in man with the 133Xenon wash out technique *Scand J clin Lab Invest* 1966 *18* 299—315
LASSEN N A On the theory of the local clearance method for measurement of blood flow including a discussion of its application to various tissues *Acta med scand* 1967 Suppl 472 136—145
LASZT L and A MULLER Der myokardiale Druck *Helv physiol pharmacol Acta* 1958 *16* 88—106
LINDER E Measurements of normal and collateral coronary blood flow by close arterial and intramyocardial injection of Krypton85 and Xenon133 *Acta physiol scand* 1966 *68* Suppl 272 5—31
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References

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Acta physiol scand 1969 76 385—392

From the Department of Radiotherapy University Hospital Lund and the Department of Zoophysiology University of Gothenburg Sweden

Secretive Response of the Electrically Stimulated Nasal Salt Gland in Larus Argentatus (Herring Gull)

By

C H HÅKANSSON and B MALCUS

Received 30 April 1968

Abstract

HÅKANSSON C H and B MALCUS *Secretive response of the electrically stimulated nasal salt gland in Larus argentatus (Herring Gull)* Acta physiol scand 1969 *76* 385—392

The secretion from the nasal salt gland in *Larus argentatus* (Herring Gull) was studied by a new type of flowmeter which allowed the secretion at electrical nerve stimulation to be followed continuously The maximal secretory capacity of the gland was found to be within the range of 280—375 $mm^3/min/g$ gland Equal volumes and equal electrolytic concentrations were secreted from the two ducts of the gland After the period of stimulation the secretion continued at a decreasing rate for about 2 min This afterflow was supposed to be the result mainly of post synaptic afterdischarge and physical distension of the gland

Marine birds have glands in their head (Technau 1936) which upon salt loading serve the osmoregulation by secreting sodium chloride in highly hypertonic solutions (Fange *et al* 1958) These salt glands *glandulae nasales* (Schmidt Nielsen 1960) as a means for desalination are surpassed only by the most efficient mammalian kidneys Their biological importance is evident from the fact that the avian kidney at its best is only able to secrete half of the electrolytic concentrations in seawater (Schmidt Nielsen 1960)

The activity of the salt gland in *Larus argentatus* is presumably controlled by nerves (Fange *et al* 1958) but corticosteroid hormones seem to be necessary for the proper function of the gland (Holmes *et al* 1961) Pharmacological observations indicate that the secretory nerve fibres are parasympathetic (Fange *et al* 1963) However functional and anatomical details of the innervation are still insufficiently known

Observations on flow rates and electrolytic concentrations from the stimulated gland have been reported (McFarland 1959 Schmidt Nielsen 1964) The use however of a more advanced recording technique which permits continuous observ of the flow rate seems to be valuable since it simultaneously gives informa

the secretive capacity of the salt gland The purpose of the present investigation is, therefore, to give an account of the flow rates and electrolytic concentrations in the electrically stimulated salt gland as measured by a new experimental device and to compare the data with those published earlier

Material

Young birds (1—2 years of age, body weight 780—1000 g) of *Larus argentatus* were trapped and kept in captivity from 18 hours to one week They were fed wastes of fish and bread as well as natural seawater *ad libitum* Totally 15 birds were used

Methods

Anesthesia An initial dose of 1 0 ml/kg b w of Mebumal sodium (6 %) in the femoral muscle resulted in deep anesthesia after 30 min at most This was maintained for 8—10 hrs by 0 1—0 2 ml every 20 min

As secretory centres in the CNS are blocked by anesthesia no reflexely induced secretion occurs in the experimental birds (Fange *et al* 1958)

Operation technique The operation was performed according to Fange *et al* (1958)

Anatomical considerations Parasympathetic secretory nerve fibres in r *palatinus* of the facial nerve run parallel with r *ophthalmicus* of the trigeminal nerve 5—7 mm in the anterior upper part of the orbit Numerous synapses occur along the common nerve and this ganglionic mass is called *ganglion ethmoidale* (Cords 1904) or *ganglion sphenopalatinum* (Webb 1957) In order to relate the position of the stimulation electrodes to these synapses the nerve was cut in 10 μ sections stained with hematoxylin and eosin and the number of ganglionic cells calculated as shown in Fig 1

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The cut ends of the opthalmic and palatine rami were tied together and pulled into a plastic tube in order to isolate them and stimulation needle electrodes penetrated into the plastic tube lumen the cathode being at the distal part of the nerve

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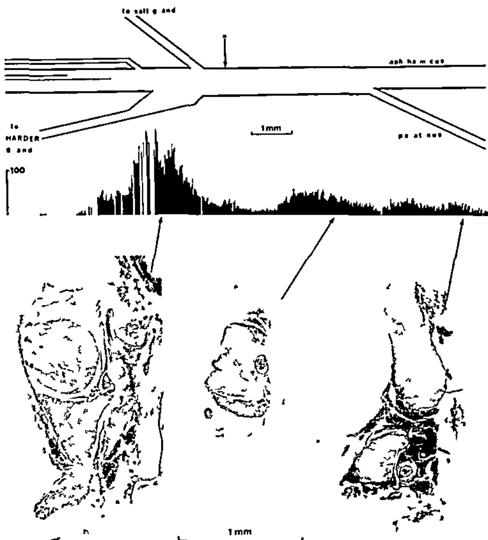


Fig 1 *Upper figure* Diagram of *ganglion ethmoidale* with nerve fibres to the salt gland from *ramus palatinus* of the facial nerve (dotted lines)
Ganglionic cells number/10 μ sections) divide into two distinct ve masses along the common nerve
Lower figure Photographs of sections (indicated by arrows) show dark ganglionic cells

resulted in a latency of 16—20 sec which is the time latency (Thesleff and Schmidt Nielsen 1962) for the advance of the ink fluid At a pressure of 5 mm H_2O the counterforce of the ink was overcome and further secretion did not cause any measureable rise in the air compression

Electrolyte concentrations Secretory samples were cold stored for at most two days in sealed plastic tubes They were analysed for sodium and potassium ions with an Eppendorf flamephotometer also used in plasma and seawater analyses

Blood samples were taken n a c aneo ve n at the tibiotarsal joint and centrifuged immediately for 5 min at 6500 rpm

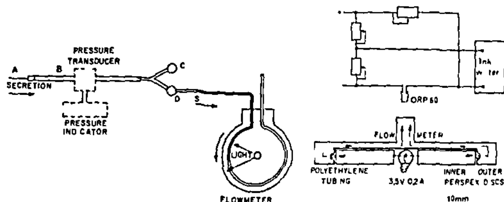


Fig. 2A. *Upper figure.* Phototube with its electrical circuit and ink writer.
Lower figure. Flowmeter (side view) suitable for measuring slow rates. Broad contour marks the area impermeable to light. The arrows show the light path through the homogenous perspex material. Between the inner and outer discs the circles simulate the perspex tubing containing the ink column.

Fig. 2B. Principle of the flow measuring when an air volume is left between secretion and ink column. The compression of the air bubble is measured by a pressure transducer (Sanborn 268B.)

A Plastic tubing in the gland duct
B Plastic tubing containing air
C, D Taps
S Ink column

Results

Flow measurements. The appearance of the continuous secretionary curve upon nerve stimulation is shown in Fig. 3. The upper curve (a) is from the medial and lateral ducts together. In this case a catheter of 0.35 mm o.d., 0.15 i.d. was used in both ducts. Stimulation was 30 c/s and 5 V for 3 min. Latency is about 15 sec. The slope of the curve during the last 2/3 of the stimulation period is practically linear and indicates a flow rate of 2 µl/sec. From the end of the stimulation the outflow from the ducts continues at a slowing rate for about 3 min.

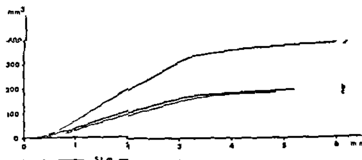


Fig. 3. Flow recording from
a lateral and medial duct together
b medial duct
c lateral duct
Stim. 30 cps 1 msec 5V

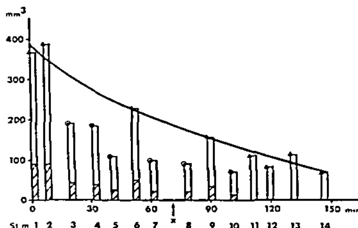


Fig 4 Recording of secreted volume during repetitive stimulation, each stimulation period being 3 min

□ —secretion during stimulation
□ =afterflow
▲ —recording made from lateral and medial duct
○ —recording made from medial duct
● =recording made from lateral duct

At x 20 ml artificial seawater is given orally
(Na 430 meq/l K 9 meq/l)

The lower curves (b and c) are from the medial and lateral duct respectively, the duct not measured having been left catheterized but open Stimulation was performed at 10 min intervals The two curves have mainly practically the same latency, namely 16—18 sec and a rise of about 1—1 1 µl/sec The total production of secretion was 190 µl for a 3 min stimulation period from the medial duct and 185 µl from the lateral duct or together 375 µl nearly equalling the amount from both ducts (387 µl) in the first experiment This indicates that the same volume of secretion is delivered from each part of the gland This was originally proposed by Fänge *et al* 1958) but has yet not been proved

In long term experiments (hours) at frequently repeated stimulations decreasing volumes were obtained In extreme cases the decrease could be as much as shown in Fig 4 Possibly this could be due to the extraordinary function of this gland *i e* to secernate a solution of sodium chloride six times more concentrated than that of blood plasma Another explanation is the deprivation of the blood supply to the ganglionic cells when the nerves were cut at the operation An oral salt dose was not able to hinder the decrement

At the end of the stimulation the flowmeter registered further flow from the duct afterflow The amount was found to be in direct relation to the rising rate of the flow curve (Fig 5a and b) In two birds the flow of which was recorded from both the medial and the lateral duct the correlation between the afterflow and the flo rate was found to have a straight line relationship (Fig 5a) The correlation bet

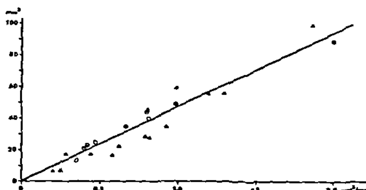


Fig 5A. Volume of afterflow (ordinate) plotted against the flow rate (abscissa). Values from two birds. Recording made from

●▲ = medial and lateral duct together
○△ = lateral duct, the medial duct catheterized but open
⊙▽ = *medial duct the lateral duct catheterized but open*

the same parameters for the medial and lateral duct in the same birds was best fitted by the same line having a slope of 47.3 μl/μl/sec. In these curves both ducts are [illegible] by a catheter of 0.35 mm o.d. 0.15 i.d. the duct not measured having been left catheterized but open.

In two more experiments only the secretion from the lateral duct was measured while the opening of the medial duct was left intact. The relation between the afterflow and the flow rate in these cases showed also the straight line relationship, the slope being 38.7 μl/μl/sec (Fig. 5b).

Maximum flow rate (*2.0* μl/sec) was registered for a gland weight of *0.31* g representing an output of 375 μl/min/g gland tissue. The value was obtained from the whole gland (measured by both the medial and the lateral ducts). From the lateral duct 0.93 μl/sec was the highest value. This was obtained from another gland of

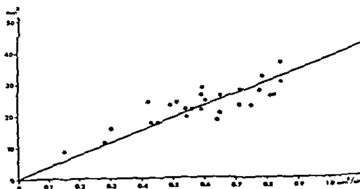


Fig. 5B. Volume of afterflow (ordinate) plotted against flow rate (abscissa). Values from two birds.

(*●) Recording made from the lateral duct, the medial duct uncatheterized and open

TABLE I Maximum secretory capacity from the nasal salt gland at nervous stimulation

Animal no	Gland weight g	Recorded duct lateral L medial M	Max flow rate µl/sec	Max spec secretive capacity µl/min/g gland
1	0.25	L	0.60	288*
2	0.32	L	0.82	307*
3	0.33	L	0.87	316*
4	0.32	L	0.84	315*
5	0.32	L	0.93	360*
6	0.27	L	0.63	280*
7	0.29	L + M	1.80	372
8	0.30	L + M	1.85	370
9	0.31	L + M	2.00	375

* Calculated as twice the flow rate from the one duct

secretory capacity for this gland was ~360 µl/min/g if the values are extrapolated to be valid for a whole gland. That such an extrapolation is tolerable is shown by the experiment presented in Fig. 4, where about the same amounts of fluid were obtained from each duct as a result of equivalent stimulations. Mean value of the maximal specific secretory power from nine birds was 330 µl/min/g (Table I).

Electrolytic concentrations. Normal values in the experimental series were for Na 690—910 meq/l and for K 44—63 meq/l, which is approximately the same values as those given in the literature (Schmidt-Nielsen 1964). The medial and lateral duct delivered the same concentrations.

In Fig. 4 it was shown that the secreted volumes diminished at repeated stimulations. The electrolytic concentration, however, were unchanged.

Discussion

The main purpose of this paper is to draw attention to some physiological phenomena of the nasal salt gland not earlier dealt with, which can be measured by a type of flowmeter described above. The secretory capacity of the gland was measured from both medial and lateral ducts in three experiments and was found to be 372, 370, and 364 µl/min/g gland. This is about double the value of that measured from the lateral duct only. This confirms the conclusion of Fange *et al.* (1958) that 50 % of the outflow is secreted from each duct.

As compared with the human glomerular filtration rate of 0.2 ml/min/g kidney (Nechay *et al.* 1960) and 0.03 ml/min/g kidney in maximum water diuresis (Schmidt-Nielsen 1960) this salt gland output of 0.3—0.4 ml/min/g is very high. If such values were to be obtained from a stimulation induced by osmosis they would correspond to an i.v. salt load of 10 ml/kg b.w. of 10 % NaCl (Fänge *et al.* 1958). Minimum i.v. salt load to induce gland secretion in gulls seems to be about 0.5 ml 10 % NaCl/kg b.w. (McFarland 1964).

The secretion of fluid per unit time was found to accelerate during the first part of stimulation up to a steady state registered as a linear slope by the flowmeter (Fig 3). To attain this linearity the gland has to build up a constant overpressure which as calculated from the slope is in the range of 30—60 mm H_2O. After the cessation of the stimulus the flow of secretion continued at a decreasing rate for about 2 min.

These findings might indicate the existence of an afterdischarge since the stimulation could not be performed completely postsynaptically (Fig 1). On the other hand, a physical distension of the gland during secretory activity is also able to contribute to the afterflow rate.

References

Cords, E. Beiträge zur Lehre vom Kopfnervensystem der Vögel. *Anat. Hefte* 1904 26 49—100
Fänge, R., K. Schmidt-Nielsen and M. Robinson. Control of secretion from the avian salt gland. *Amer. J. Physiol.* 1958 195 321—326
Fänge, R., J. Krog and O. Reite. Blood flow in the salt gland studied by polarographic oxygen electrodes. *Acta physiol. scand.* 1963 58 40—47
Holmes, W. N., D. G. Butler and J. G. Phillips. Observations on the effects of maintaining glaucous-winged gulls (Larus Glaucescens) on fresh water and sea water for long periods. *J. Endocr.* 1961 23 53—61
McFarland, L. Captive marine birds possessing a functional lateral nasal gland (Salt Gland). *Nature, Lond.* 1959 184 2030—2031
McFarland, L. Minimal salt load required to induce secretion from the nasal salt glands of sea gulls. *Nature, Lond.* 1964 204 1202—1203
Nechay, B. R., J. L. Larimer and T. H. Maren. Effect of drugs and physiologic alterations on nasal salt excretion in sea gulls. *J. Pharmacol. exp. Ther.* 1960 130 401—410
Schmidt-Nielsen, K. The salt-secreting gland of marine birds. *Circulation* 1960 21 955—967
Schmidt-Nielsen, K. *Physiology of salt glands.* 2. Wissenschaftliche Konferenz der Gesellschaft deutscher Naturforscher und Ärzte 1964. Springer Verlag Berlin, Heidelberg, New York 1965 pp 269—287
Technau, G. E. Die Nasendrüse der Vögel. *J. f. Ornithol.* 1936 48 511—617
Thesleff, S. and K. Schmidt-Nielsen. An electro-physiological study of the salt gland in the herring gull. *Amer. J. Physiol.* 1962 202 597—600
Webb, M. The ontology of the cranial bones, cranial peripheral and cranial parasympathetic nerves, together with a study of the visceral muscles of Struthio. *Acta Zool. Stockh.* 1957 38 1—203

Acta physiol. scand. 1969. 76. 393—395

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Urinary Excretion of Catecholamines and Vanilmandelic Acid in Rats Exposed to Cold

By

ION MOTELICA[1]

Received 26 September 1968

Abstract

MOTELICA I *Urinary excretion of catecholamines and vanilmandelic acid in rats exposed to cold* Acta physiol scand 1969 *76* 393—395

Male Sprague Dawley rats of an average weight of 300 g excreted during laboratory conditions at 20—22° C on an average 18 ng/kg/hr adrenaline (A), 97 ng/kg/hr noradrenaline (NA) and about 4 µg/kg/hr vanilmandelic acid (VMA) Individual, diurnal and seasonal

Urinary excretion of catecholamines during exposure to cold has been studied both in animals (Johnson 1963, Johnson 1966 Le Blanc *et al* 1967, Leduc 1961, Pouliot 1966) and in man (Arnet and Watts 1960)

It was generally observed that NA reaches maximum values in the first hours and remains at a high level during the entire duration of exposure Adrenaline excretion increases gradually to a maximum value within about one week, thereafter gradually decreasing No data seem to be available concerning the excretion of VMA during exposure to cold

The main role in the adaptation to cold and survival is attributed to NA while A is considered only as the secondary line of defense (Leduc 1961)

Since it seemed of interest to follow also the excretion of VMA in urine during exposure of rats to cold in order to obtain some more information of the total catecholamine release this has been studied in addition to the excretion of free catecholamines

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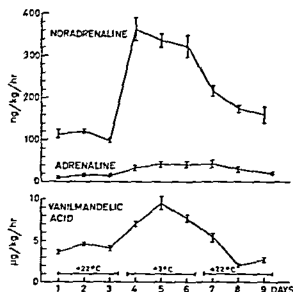


Fig 1 Adrenaline noradrenaline and vanilmandelic acid urinary excretion in rats prior to during and after exposure to the temperature of +3° C.

Materials and methods

The experiments were carried out during March-October on male Sprague Dawley rats weighing on an average 300 g purchased from Anticimex, Stockholm.

After an adaptation of at least 3 days to laboratory conditions (temperature 20—22° C water and food *ad libitum*) lots of 6 rats each of the same weight were placed in individual metabolic cages These were provided with plastic funnels and metallic grates covered with polyvinylic paint. Faces and food were separated from the urine by a glass trap system Urinary pH was adjusted to 3—4 by the initial addition of 5 ml 1 N acetic or sulphuric acid into the collecting vessels Urine was generally collected for 24 hrs. At the end of the 24 hr period a slight pressure of the abdomen was exerted in order to induce voiding The rats were then moved into clean cages urine being collected for a new period. The funnels were washed with acidulated distilled water which was added to the respective samples of urine These were centrifuged and the free catecholamines determined fluorimetrically according to Euler and Lishajko (1961 The VMA was measured by the method of Pisano *et al* (1962

The values obtained were expressed in ng/kg/hr for catecholamines and in μg/kg/hr for VMA. The standard error (SEM) and the "t" test were computed

All values are given in terms of the base or acid but uncorrected for the recovery which was about 75 per cent

Results

1 *Urinary excretion of catecholamines and VMA in normal conditions*

Individual variations In our material the rats excreted 18 ± 1 9 ng/kg/hr of A 97 ± 4 1 ng/kg/hr of NA 4 2 ± 0 1 μg/kg/hr of VMA

Diurnal variations In one and the same animal the values during different days varied between 12—23 ng/kg/hr for A 85—99 ng/kg/hr for NA and 3 2—5 0 μg/kg/hr for VMA or between approximately the same limits as for the individual variations

2. *Urinary excretion of catecholamines and VMA during exposure to cold*

The experiments were carried out in the months of July and September No significant differences between the results were noted and the values for the two groups have been treated together

After a period of 3 days at room temperature the rats were exposed to an ambient temperature of $+3°$ C for 3 days and then brought back to room temperature The results are presented in Fig 1

As observed in earlier studies (Leduc 1961) NA excretion reached a maximum the first day and remained at this high level throughout the duration of exposure Adrenaline increased more gradually

In the case of VMA the increase reached its maximum value only after 2 days, thereby differing from NA

When the animals were brought back to a temperature of $+20$—$22°$ C, the values returned gradually to normal, somewhat more rapidly for NA than for A and VMA (Fig 1)

Comment

The present results on catecholamine excretion during exposure to cold confirm these obtained by Leduc (1961), and also show that the excretion of VMA is increased during these conditions

While NA excretion increased 3 to 4 times during exposure to cold the VMA excretion was about doubled, suggesting that during higher excretion rates of NA the proportion of free amine in the urine increases This observation is in keeping with the data obtained for adrenaline by Bygdeman Euler and Hökfelt (1960)

It is of interest to note that while the NA increases rapidly during exposure to cold, the VMA only slowly increases, reaching a maximum on the second day VMA thus less faithfully reflects the time-course of the changes occurring in the catecholamine release in the organism

The research reported in this document has been sponsored in part by the AIR FORCE OFFICE OF SCIENTIFIC RESEARCH under Grant EOOAR-68-0009 through the European Office of Aerospace Research (OAR), United States Air Force

References

Arnet E L and D T Watts Catecholamine excretion in men exposed to cold *J appl Physiol* 1960 *15* 499—500

Bygdeman S L S v Euler and B Hokfelt Resynthesis of adrenaline in the rabbit s adrenal medulla during insulin induced hypoglycemia *Acta physiol scand* 1960 *49* 21—28

Euler, U S v and F Lishajko Improved technique for the fluorimetric estimation of catecholamines *Acta physiol scand* 1961 *51* 348—355

Johnson G E The effect of cold exposure on the catecholamine excretion of adrenalectomized rats treated with reserpine *Acta physiol scand* 1963 *59* 438—444

Johnson D G The effect of cold exposure on the catecholamine excretion of rats treated with decaborane *Acta physiol scand* 1966 *68* 129—135

LeBlanc J A D Robinson D F Sharman and P Tousignant Catecholamines and short term adaptation to cold in mice *Amer J Physiol* 1967 *213* 1419—1422

Leduc J Catecholamine production and release in exposure and acclimation to cold *Acta physiol scand* 1961 *53* Suppl 183

Pisano J J J R Crout and D Abraham Determination of 3 methoxy-4 hydroxymandelic acid in urine *Clin chim Acta* 1962 7 285—291

Poulliot M Catecholamine excretion in adreno-demedullated rats exposed to cold after chronic guanethidine treatment *Acta physiol scand* 1966 *68* 164—168

Acta physiol scand. 1969. 76 396—398

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Compensatory Hypertrophy of the Rat's Submaxillary Gland

By

MATS ELMER and PER OHLIN

Received 1 October 1968

Abstract

ELMER, M and P OHLIN *Compensatory hypertrophy of the rat's submaxillary gland* Acta physiol scand 1969 76 396—398

The secretory activity of the submaxillary gland in the conscious rat is increased in the acute experiment when the secretion from one or more of the other salivary glands is prevented from reaching the oral cavity In chronic experiments the weight of the submaxillary gland is augmented after duct ligation of other salivary glands It seems likely that increased cretory activity as observed in the acute experiments, is the cause of this glandular hyper phy

The weight of salivary glands seems to be dependent on their activity Thus in rats it is reduced when the animals are on a liquid diet (Hall and Schneyer 1964 Wells and Peronace 1967) which is supposed to decrease the secretory activity Similarly the size of the submaxillary glands is diminished after section of the sympathetic or parasympathetic glandular nerves (see Ohlin 1966 a) which also reduces the secretory activity since both sets of nerves have been shown to elicit secretion from the glands in conscious rats (Ohlin 1968) On the other hand treatment with a sialagogue agent e g pilocarpine abolishes the atrophy following denervation (Ohlin 1966 b)

The present experiments were carried out to study the relation between organ activity and glandular weight In one group of rats the secretory activity of the submaxillary gland was estimated in conscious animals when stimulating secretion reflexly by oral application of citric acid The flow of saliva was studied in controls and after duct ligation of one or more of the other major salivary glands It was found to increase when the secretion from some other glands was prevented from reaching the oral cavity In another group of rats the effect of this increased secretory activity on the weight of one submaxillary gland was determined 3 weeks after duct ligation of other major salivary glands the size of the submaxillary gland was found to be augmented

Methods

Female rats of a strain bred at this Institute were used when aged about 4 months. The animals were given a pelleted diet (Anticimex 210) and water *ad libitum*

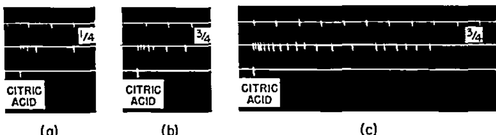


Fig 1 Secretory responses of the rat s submaxillary gland after citric acid (a) No ducts ligated (b) the parotid ducts ligated (c) the submaxillary and sublingual ducts ligated as well Records from above time in min drops of saliva signal

To study the secretory responses of the submaxillary gland to citric acid 12 rats were used Under ether anaesthesia a thin polythene tube was inserted into the submaxillary duct as far cranial as possible to avoid damage to the chorda lingual nerve The tube was fastened by two l gatures and a collar was put round the neck to prevent the rat from pulling out the cannula The animal was then placed in a metal cage and drops of saliva appearing at the tip of the cannula were marked on a smoked drum In the conscious animals, about 15 min after the operation the secretory responses to citric acid (0 1 ml 100 mg/ml) were estimated All determinations were made 3 times in each rat and the mean was calculated Immediately [illegible] ligation of the two parotid ducts [illegible] ducts (6 rats) The secretory re [illegible] 4 rats the ducts of the remaining [illegible] were then ligated and the meas [illegible] ulation procedure alone prevented the secretion of the submaxillary and sublingual glands on the cannulated side from reaching the oral cavity

In 16 rats the weight of the submaxillary glands was estimated in controls in one group of rats after ligation of the submaxillary and sublingual ducts on one side and in another group of animals after ligation of both parotid ducts as well Three weeks later the rats were killed by cervical dislocation and the submaxillary glands were carefully dissected and cleaned The weight was determined as wet weight and as dry weight after heating to 110° C for 48 hrs

Results

The secretory response of the submaxillary gland to the standardized solution of citric acid was found to be 4 1 ± 0 36 (12)[a] drops of saliva when the submaxillary and sublingual glands on the contralateral side and the two parotid glands were still secreting into the oral cavity Ligation of the parotid ducts increased the secretory response significantly ($P<0\ 01$) from 4 1 ± 0 36 (12) to 6 9 + 0 96 (6) drops of saliva (Fig 1) The secretory response seemed also slightly increased ($0\ 05<P<0\ 1$) to 6 ± 1 4 (6) drops of saliva after ligation of the submaxillary and sublingual glands on the contralateral side The secretory responses to citric acid appeared when the rat started licking movements and they were finished within a few min On the other hand a longlasting profuse secretion 25 ± 4 6 (4) drops of saliva was noticed when the secretion from all major salivary glands was eliminated (Fig 1)

Ligation of the submaxillary and sublingual ducts on one side and the two parotid ducts caused a significant ($P<0\ 01$ increase in weight of the intact submaxillary

[a, b] Mean ± standard error of mean (number of [a] rats or [b] glands)

gland in 3 weeks, by about 20 % Thus, the dry weight increased from 31 8 ± 0 83 (10)[1b] mg to 38 ± 1 4 (6) mg, the wet weight was similarly increased from 131 ± 2 2 (10) mg to 159 ± 4 1 (6) mg The dry weight of the duct ligated submaxillary gland was markedly decreased, from 31 8 ± 0 83 (10) mg to 10 ± 1 7 (6) mg in accordance with previous observations (Junqueira and Rabinovitch 1954, Ohlin and Perec 1967)

After ligation of the submaxillary and sublingual ducts on one side alone, the weight of the remaining submaxillary gland was slightly increased, by about 4 %

Discussion

The secretory response of the rat's submaxillary gland to citric acid was found to increase when saliva from the other major salivary glands was prevented from reaching the oral activity It was slightly increased after duct ligation of the contra lateral submaxillary and sublingual glands and significantly augmented after duct ligation of the two parotids These findings indicate that the salivation from the two parotid glands remove the citric acid from the mouth more effectively than the secretion from one submaxillary and sublingual gland The longlasting flow of saliva when no secretion from the major salivary glands reached the oral cavity indicates that the secretion from glandular cells in the mucous membranes of the mouth is scanty and of minor importance which has been suggested previously (Wallenius 1966)

There seems to be a close relationship between the secretory activity and the size of the submaxillary gland in rats Thus, in the acute experiment the secretory activity of the gland was significantly increased when the secretion from one submaxillary and sublingual gland and the two parotid glands was prevented from reaching the oral cavity the weight of the gland was increased under similar conditions in 3 weeks The present results support the previous suggestion (Hall and Schneyer 1964) that an increased secretory activity is followed by glandular hypertrophy They are also in accordance with similar findings on the weight of glands after extirpation of salivary glands (Schwartz and Shaw 1955)

References

HALL, H D and C A SCHNEYER Salivary gland atrophy in rat induced by liquid diet *Proc Soc exp Biol* (N Y) 1964 *117* 789—793

JUNQUEIRA L C and M RABINOVITCH Reversibility of the phenomena induced by excretory duct ligature in the rat submaxillary gland *Tex Rep Biol Med* 1954 *12* 94—97

OHLIN P Nervous and hormonal control of salivary glands in rats *Acta Univ Lund II* 1966 a *7* 1—21

WELLS H and A A [illegible] glands of rats *Amer J Physiol* 1967 *212* 247—251

Acta physiol. scand 1969. 76 399—405

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A Procoagulant from Human Postpartum Serum

Effects on coagulation and hemodynamics in dogs

By

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Received 7 October 1968

Abstract

NORDSTROM S, P OLSSON, A C TEGER NILSSON and E ZETTERQVIST *A procoagulant from human postpartum serum Effects on coagulation and hemodynamics in dogs* Acta physiol scand 1969 *76* 399—405

Several investigations have been made on the effects of different tissue thromboplastins on coagulation *in vivo*, and the ability of tissue thromboplastin to induce intravascular coagulation is well documented (*cf* Penick and Roberts 1964, McKay 1965) Tissue thromboplastin from placenta has been studied extensively (Copley 1945 Fulton and Page 1948 Page *et al* 1951, Schneider 1947), and it has been suggested that some bleeding and thrombotic complications in late pregnancy may be due to leakage of placental tissue thromboplastin into the maternal blood stream (Schneider 1950) Recently human postpartum serum has been shown to contain a procoagulant which behaves like tissue thromboplastin *in vitro* (Teger Nilsson 1967 a b)

The purpose of this investigation was to study the effect *in vivo* of the procoagulant from human postpartum serum (PPS) and to see whether, like other tissue thromboplastins it does induce intravascular coagulation PPS was therefore injected into dogs and its effect on fibrinogen and prothrombin concentrations platelet count and elimination of labelled fibrinogen was studied The effects were compared with those of homologous brain thromboplastin given to dogs in a similar study Nordstrom and Zetterqvist (1969 In addition aortic blood pressure and fe arterial blood flow were recorded

TABLE I Effects of PPS on coagulation factors The fibrinogen radioactivity is expressed in per cent of the preinjection value which is arbitrarily set at 100 per cent

Dog no	Concentration of	Before anaesthesia	Before PPS	Hours after PPS injection					
				1/4	1/2	1	2	4	6
1	Fibrinogen, mg/100 ml plasma	0 53	0 50	0 39	0 37	0 33	0 30	—	—
	Fibrinogen radioactivity %	107	100	89	73	64	57	—	—
	Prothrombin, NIH u of formed thrombin/ml plasma	42	40	28	27	22	20	—	—
	Platelets number/nl blood	460	450	6	13	66	82	—	—
	Hematocrit %	47	40	42	41	43	50	—	—
2	Fibrinogen	0 51	0 45	0 29	0 30	0 29	0 28	—	—
	Fibrinogen radioactivity %	116	100	60	59	61	52	—	—
	Prothrombin	51	47	26	28	28	25	—	—
	Platelets	480	470	11	24	56	74	—	—
	Hematocrit %	*43*	*33*	*38*	*39*	*38*	*38*	—	—
3	Fibrinogen	0 71	0 65	0 68	0 76	0 61	0 63	0 65	0 53
	Fibrinogen radioactivity %	113	100	91	102	89	90	88	70
	Prothrombin	42	39	31	35	31	27	31	22
	Platelets	240	250	12	11	74	110	180	200
	Hematocrit %	40	33	37	36	3[illegible]	40	35	38
4	Fibrinogen	0 30	—	0 22	0 18	0 20	0 19	0 18	0 18
	Fibrinogen radioactivity %	100	—	75	71	71	73	70	77
	Prothrombin	—	—	—	—	—	—	—	—
	Platelets	290	—	39	67	81	73	110	190
	Hematocrit %	47	—	46	40	38	38	39	37

per cent of the preinfusion value within 9—21 min In one animal the low pressure persisted throughout the experiment

The heparinized animals when injected with PPS also evidenced a fall in blood pressure from 140—180 mm Hg to 91—145 mm Hg The pressure reached 90 per cent of the preinjection value in 1—6 min

Infusions of PPS into the femoral artery produced no major changes in the femoral arterial blood flow indicating that PPS has no direct action on the peripheral b'ood vessels and was not contaminated with vasoactive material

Discussion

Conversion of prothrombin to thrombin and of fibrinogen to fibrin is inevitable in thromboplastin induced coagulation Consequently the concentrations of prothrombin and fibrinogen in the blood were followed The platelet count was followed as being a sensitive indicator of intravascular coagulation Previous studies on the elimination rate of iodine labelled fibrinogen have given valuable information on

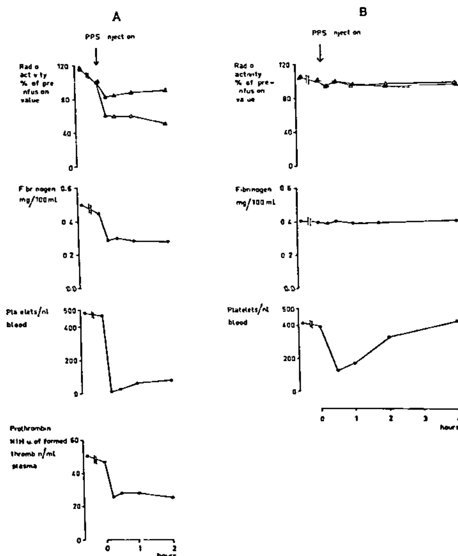


Fig 1 A Effects of PPS injections on coagulation factors in one dog
▲——▲ plasma radioactivity △——△ fibrinogen radioactivity – – – – – elimination rate of labelled fibrinogen in anaesthetized unheparinized dogs with an indwelling catheter (cf Zetterqvist *et al* 1968)
B Effect on coagulation factors in one dog when 1000 u of heparin per kg b w was given prior to the PPS injection

thromboplastin induced intravascular coagulation especially with respect to its relation to fibrinolysis (Nordstrom and Zetterqvist 1969) The same methode were therefore applied

The results of the present study should be compared to the results of a previous investigation on thromboplastin induced intravascular coagulation (Nordström a Zetterqvist 1969) In this study dogs were given homologous brain thrombopl

with the same technique but in somewhat higher doses The mortality rate was comparatively high in both groups Injection of PPS and homologous brain thromboplastin gave rise to similar changes in fibrinogen concentration, fibrinogen radioactivity and platelet count In the present study also prothrombin concentration was measured and found to decrease The results presented should moreover be evaluated with respect to the findings in dogs treated with anesthesia, saline and indwelling catheters (Zetterqvist *et al* 1968) These dogs showed no changes in fibrinogen concentration and platelet count but a slightly increased elimination rate of iodine labelled fibrinogen The latter finding is, however, of minor importance for the present investigation In view of this background it is assumed that PPS, like homologous brain thromboplastin induces consumption of coagulation factors The observation that PPS injection into a heparinized dog was without effect on fibrinogen strengthens this assumption

Fibrinolysis in connection with intravascular coagulation has been reported both after thrombin and thromboplastin infusions (Kowalski *et al* 1965, Nordstrom and Zetterqvist *1968* Seegers *1961) In this study as in the others fibrinolysis* was indicated by an increasing difference between plasma and fibrinogen radioactivity Fibrinolysis, following the injections of PPS seems to be variable

Since hemodynamic changes are an important manifestation of intravascular coagulation (Hardaway 1966 McKay 1965) the circulatory effects of PPS seem to be of interest I v injections of PPS produced a pronounced drop in blood pressure The intraarterial infusions of the substance however induced no changes in the local peripheral blood flow indicating that PPS does not contain any important amounts of vasoactive material

Several mechanisms are probably involved in the PPS induced hypotension The decrease in blood pressure following injections of PPS also in the heparinized animals indicate that part of the hypotensive effect may be due to other mechanisms than intravascular coagulation It has earlier been shown that thrombin in concentrations as low as 0 01—1 NIH u/ml blood exerts peripheral vasodilatation and hypotension (Delin *et al* 1967 Olsson *et al* 1969) As judged from the decrease in prothrombin concentration as much as 10—20 NIH u of thrombin per ml plasma might have been formed in the present experiment It is therefore reasonable to conclude that part of the hypotension is secondary to activation of the plasma coagulation system with thrombin formation and subsequent peripheral vasodilatation A decrease in the cardiac output as a consequence of thrombus formation especially in the pulmonary circulation must also be taken into consideration as a cause of the decrease in blood pressure

In conclusion intravenous injections of PPS induce consumption of coagulation factors and hypotension findings which indicate intravascular coagulation The present results are in agreement with those obtained with other tissue thromboplastins

This investigation was aided by grants from Karolinska Institutet Adolf Robberts foundation Svenska Sallskapet for Medicinsk forskning and Swedish Medical Research Council no K67 19X 520 03

We wish to express our thanks to Laborator Birger Blombäck and to Laborator Erik Berglund, for valuable discussions during this work and to Miss Bodil Jonsson and Miss Ingrid Söderman for skilful technical assistance

References

BERGSTROM, K, B BLOMBACK and G KLEEN, Studies on the plasma fibrinolytic activity in a case of liver cirrhosis *Acta med scand* 1960 *168* 291—305

BIGGS, R and R G MACFARLANE, *Human blood coagulation and its disorders* 1962 3rd Ed. p 385 Blackwell, Oxford

BLOMBACK, B and M BLOMBACK, Purification of human and bovine fibrinogen *Arkiv Kemi* 1956 *10* 415—443

BLOMBÄCK, B, M BLOMBÄCK and E HOLMBERG, A new method for fractionation of proteins *Acta chem scand* 1966 *20* 2317—2319

COPLEY, A L, Studies on human placental thromboplastin in vitro and in vivo *Science* 1945 *101* 436—437

DELIN, A, P OLSSON and A-C TEGER NILSSON, Vasodilatation in the canine leg caused by intra arterial infusion of thrombin and tissue thromboplastin *Cardiovasc Res* 1967 *1*, 371—378

FULTON, L D and E W PAGE, Nature of the refractory state following sublethal dose of human placental thromboplastin *Exp Biol Med* 1948 *68* 594—596

HARDAWAY, R. M, *Syndromes of disseminated intravascular coagulation* With special reference to shock and hemorrhage Thomas Publisher Springfield, Ill U S A 1966

KOWALSKI, E, A Z BUDZYNSKI, MARIA KOPEC, Z S LATALLO, B LIPIŃSKI and Z WEGRZYNOWICZ, Circulating fibrinogen degradation products (FDP) in dog blood after intravenous thrombin infusion *Thrombos Diathes haemorrh* (Stuttg) 1965 *13* 12—24

KRISTENSSON, A, A new method for the direct counting of the so called blood platelets in man *Acta med scand* 1922 *57* 301—312

LEANDOER, L, L APPELGREN and S-E BERGENTZ, Fibrinogen turnover after massive hemorrhage in dogs studied with radioactively labelled fibrinogen *Acta chir scand* In press

LEWIS, J, E FERGUSON and C SCHOENFELD, Studies concerning the turnover of fibrinogen I^{131} in the dog *J Lab clin Med* 1961 *58* 247—258

MCFARLANE, A S, Labelling of plasma proteins with radioactive iodine *Biochem J* 19[illegible] *62* 135—143

MCFARLANE, A S, Efficient trace labelling of proteins with iodine *Nature* [illegible] *182* 53

[illegible]

trasylol on thromboplastin-induced intravascular coagulation, studied [illegible] labelled fibrinogen *Acta physiol scand* 1969 *76* 93—105

NOREN, I and M BLOMBACK, New rapid tests for coagulation disorders [illegible] *Invest* 1965 *17* Suppl *86* 175

OLSSON, P, J SWEDENBORG and A-C TEGER NILSSON, Vasodilating [illegible] of thrombin in dogs Role of fibrinogen, plasma kinins and [illegible] *Res* 1969 In press

PAGE, E W, L D FULTON and M B GLENDENING The cause of [illegible] following abruptio placentae *Amer J Obstet Gynec* 1951 *61* 11[illegible]

PENICK, G D and H R ROBERTS, Intravascular clotting focal [illegible] *Path* 1964 *3* 269—328

SCHNEIDER, C L The active principle of placental toxin thromb[illegible] blood, antithromboplastin *Amer J Physiol* 1947 *149* 123—1[illegible]

SCHNEIDER C L *Thromboplastin complications in late pregna*[illegible] posium on Toxemias of Pregnancy Human and Veterinary, C[illegible] 181

SEEGERS W H Fibrinolytic properties of thrombin *Thrombo* [illegible] 1961 *6* 101 110

TEGER NILSSON A C A procoagulant from human postpart[illegible] terization *Scand J clin Lab Invest* 1967 a *20* 329—33[illegible]

TEGER NILSSON A C A procoagulant from human pos[illegible] properties *Scand J clin Lab Invest* 1967 b *20* 339—34[illegible]

ZETTERQVIST E L A CARLSON and S O LILJEDAHL [illegible] upon the elimination of ^{125}I fibrinogen in dogs *Act* [illegible] 1968

Acta physiol. scand 1969. 76. 406—414

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The Effect of Secretin and Pancreozymin on Pancreatic Blood Flow in the Conscious and Anesthetized Dog

By

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Received 8 October 1968

Abstract

AUNE S and L S SEMB *The effect of secretin and pancreozymin on pancreatic blood flow in the conscious and anesthetized dog* Acta physiol scand 1969 76 406—414

Pancreatic blood flow was studied in dogs by measuring local hydrogen gas clearance External pancreatic secretion was collected by cannulation for volume measurements In 16 expts (15 dogs) recordings were obtained in pentobarbital (Nembutal) anesthesia The mean value of 115 observations was 42 8 (S E 4 3) ml/min 100 g pancreas In 3 dogs measurements were made in the awake fasted state by means of chronically implanted electrodes which proved effective for about two weeks Mean of 33 observations was 76 ml/min 100 g The correlation between pancreatic blood flow and external secretion was studied by means of secretin (Boots Jorpes and pancreozymin (Boots) A variable response occurred which proved to be time dependent and not correlated to the state of secretion or stimulation An acute rise in blood flow was seen when hormone administration was started or increased This effect was immediate but transient blood flow returned to pre stimulatory level after 20 min whereas the external secretion continued unchanged No effect was noticed on gastric hydrogen clearance recorded in 4 of the dogs indicating an organ specificity of the hormonal influence

Measurement of blood flow in the pancreas has been the subject of several studies in the past However the methods employed all have shortcomings The direct methods such as Thermostrohmuhr (Gayet and Guillaume 1930 Maltesos and Watson 1939), or electromagnetic flowmeter (Eichelter and Schank 1966 MacKowiak *et al* 1967) cannot be employed without extensive surgery and only to a part of the pancreas, since the gland has its blood supply from several main trunks which also support other organs Of indirect methods plethysmography gained some interest early in this century but interpretations of blood flow changes in organ volumes proved difficult Light transmission (Hilton and Jones 1960) and heat conductance (Papp, Varga and Acs 1966) give only relative measurements of blood flow The isotope dilution technique utilizing K^{42} or Rb^{86} as introduced by Sapirstein (1958) has also been used by Delaney and Grim (1966) and by Papp *et al* (1966) This method gives a reasonable measure of the share of the cardiac output received by the pancreas but has its limitation in that only one observation can be obtained

from each animal Comparison of different states of function thus has to be performed in groups of animals

Pancreatic blood flow in resting conditions is reported in the literature to vary between 61 and 129 ml/min 100 g tissue

The hydrogen gas method for measurements of local blood flow, introduced by Aukland, Bower and Berliner (1964), was employed in the present study of pancreatic blood flow in dogs This method seemed especially suitable, since repeated measurements are possible in the same animal, whether the dog is anesthetized or not Minimal trauma is given to the organ

Blood flow was recorded in the resting state of the gland and following secretory stimulation with secretin and pancreozymin

Material and methods

Operative procedures

15 mongrel dogs weighing 12—20 kg were used The animals were fasted for 18 hrs before

elapse before measurements were started

Arterial blood pressure was recorded from a femoral artery by means of a Statham pressure transducer and a Sanborn multichannel amplifier and recorder

In 3 animals the abdomen was closed by means of interrupted silk sutures and the electrode leads were led through the abdominal wall through a separate stab wound and fastened in a specially devised holder with a bite block attached in order to protect the leads when not in use External secretion was not measured in these dogs Blood flow recordings were made in the anesthetized state and 2—12 days after the implantation of the electrodes with the dogs standing in a Pavlov frame In the awake dogs arterial cannulation was performed in local anesthesia (Lidocaine 1 % 5—10 ml s c)

The circulatory effect of secretin was tested by infusion of 1—10 I U per kg body weight Secretin was dissolved in 0 9 % NaCl immediately before use In 6 of the experiments the secretin of Boots Pure Drug Co Ltd was used and in 9 other experiments pure secretin (generously supplied by Professor E Jorpes)

Pancreozymin (Boots Pure Drug Co Ltd) was infused in doses of 1—5 clinical units per kg body weight

The majority of the observations were carried out with continuous i v administration of the hormones by means of a pump delivering the solutions at a rate of 15 ml/hr Immediate responses were tested by injections of single doses within 30—60 sec using 10—20 I U secretin and 5—15 clinical units of pancreozymin respectively

In order to determine the organ specificity of the hormones simultaneous recordings were made of pancreatic and gastric hydrogen clearance In this series including two of the awake dogs the effect of histamine and acetylcholine was investigated

Blood flow measurements

Aukland *et al* (1964 showed that local blood flow in a tissue can be measured by recording the rate of hydrogen desaturation of the tissue with platinum electrodes Hydrogen oxidation at the platinum surface generates an electrical current (i_{H_2}) which is proportional to the

l in

read from a semilogarithmic plot of the curve according to the formula F = 100 l

where $T_{1/2}$ is the half time in minutes The tissue/blood partition coefficient and specific gravity of pancreatic tissue are assumed to be 1

Hydrogen current was measured by means of a six channel polarograph and recorder (Rika denki, Kogyo, Ltd Model B64), utilizing a polarizing potential of +0 2 V versus a calomel reference electrode (For further details of the method reference is made to Aukland *et al* 1967 Aukland 1968)

The hydrogen gas was administered through the tracheal tube in the anesthetized animals giving approximately 5 % of H_2 in the respiratory air, until a fairly stable concentration was obtained in the pancreas In the conscious dogs, H_2 was provided through a thin catheter in the mouth or nose or by means of a funnel shaped device placed around the dog s snout In some of the first experiments, H_2 saturated saline was infused into the thoracic aorta for 1/2 l min after stopping the H_2-inhalation, in order to obtain a square wave drop to zero in arterial hydrogen gas concentration This procedure was abandoned, however, in later experiments since the rate of desaturation was not usually of such a magnitude as to be disturbed by the delay in arterial desaturation caused by elimination of the gas through the lungs

Results

Monoexponential desaturation curves (Fig 1) were obtained in the majority of experiments In curves deviating clearly from a monoexponential course after removal of 80 % of the hydrogen gas, the initial slope was used in determining the flow rate The results of 16 expts in 15 dogs are given in Table I, which shows pancreatic blood flow in the unstimulated state of the gland in pentobarbital anesthesia Con-

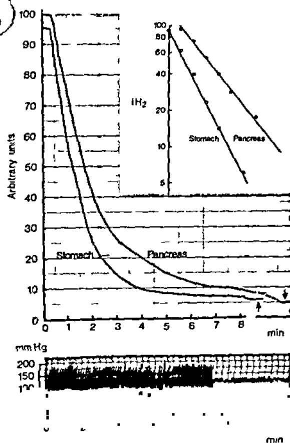


Fig 1 H_2 desaturation curves (upper left) of pancreas and gastric wall in a 15 kg awake dog (no 12) 6 days after implantation of platinum electrodes Arrows indicate asymptote Semilogarithmic plots of the curves are shown to the right The arterial blood pressure measured by cannulation through the femoral artery is shown below

TABLE I Resting blood flow in Nembutal anesthes a

Dog no	Number of desat	Number of electr	Number of obs	Mean flow in each dog	Range ml/min 100 g
1	2	4	8	16 8	12 3—19 2
2	3	1	3	20 0	19 3—20 4
3	2	6	12	32 8	17 3—49 5
4	3	3	4	33 8	30 0—38 5
5	3	2	6	34 5	26 6—42 0
6	3	4	10	42 2	23 0—92 5
7	3	5	15	49 0	23 0—73 0
8	3	3	9	49 5	38 4—58 0
9	3	4	10	60 0	26 4—107 0
10	1	4	4	20 0	16 5—24 3
10*	2	2	4	35 6	31 3—40 6
11	4	1	4	60 1	53 0—69 3
12	3	2	6	70 3	47 0—99 0
13	1	4	4	41 2	18 5—60 1
14	5	2	10	74 7	46 2—126 0
15	3	2	6	44 2	35 5—55 6
			Mean	42 8 (S E 4 3)	

* 20 days elapsed between the 2 expts in dog no 10

TABLE II Pancreatic blood flow in awake fasting dogs compared to recordings in Nembutal anesthes a

Dog No	Number of desat	Number of electr	Number of obs	Mean flow ml/min 100 g	
				Awake	Anesthetized*
4	6	3	18	62 0	33 8
12	5	3	12	117 0	70 3
15	4	2	6	49 0	44 2
Total	14	8	36	Mean 76 (S E 20 8)	49 4 (S E.10 9)

* Data taken from Table I

siderable variations were seen from one animal to another and to some extent in the different areas of the gland but no consistent difference in blood flow was found between the corpus and the uncinate process (caput) Average blood flow of 115 observations amounted to 42 8 ml/min 100 g

In conscious dogs (3 animals 36 recordings were made (Fig 1 Table II) and the average blood flow *with the animals fasted and standing in a Pavlov frame* 76 ml/min 100 g This is comparable to a mean blood flow of 49 4 ml/min

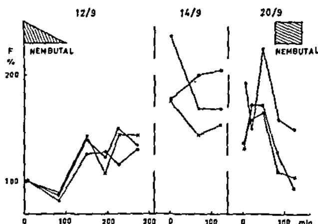


Fig 2 Effect of Nembutal anesthesia on pancreatic blood flow (F) in dog no 4 Initial value of each electrode is set at 100 per cent Electrodes implanted 12/9 and measurements continued throughout recovery from anesthesia the degree of consciousness indicated by the triangle (left) The measurements were repeated 2 and 8 days later, and terminated by a new Nembutal anesthesia

when these dogs were given Nembutal The depressive effect of Nembutal is seen in Fig 2 which also demonstrates that the electrodes were in full order when chronically implanted in the gland Recordings were obtained up to 12 days after the implantation but showed increasing delay and reduced sensitivity or were unstable after three weeks The reasons for the failure were not entirely clear at autopsy, but were probably due to leakage in the electrodes or electrical leads No infection or necrosis could be detected macroscopically but an impressive proliferation of the peritoneal tissue around the leads was apparent

Effects of hormones and vasoactive substances

The injection of secretin or pancreozymin was usually started 25—30 min before the blood flow measurements were made in order to obtain a definite external secretory response from the gland In a series of 8 anesthetized animals continuous administration of secretin gave rise to distinctly increased pancreatic blood flow in 4 animals (Fig 3) whereas no response was seen in the others As shown in Fig 4 blood flow was even reduced in some experiments during secretin stimulation However it

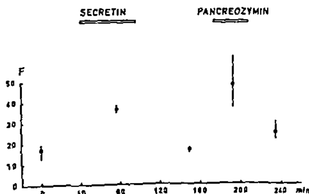


Fig 3 Pancreatic blood flow (F ml/min 100 g recorded in dog no 1 basally and during infusion of secretin and pancreozymin In this dog the resting blood flow was relatively low

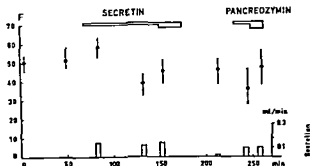


Fig 4 Pancreatic blood flow (F ml/min 100 g) and external secretion (Secretion ml/min) recorded in dog no 8 basally and during infusion of secretin and pancreozymin In this dog the resting blood flow was relatively high

seemed that in the dogs with a low resting blood flow, the response to secretin was more pronounced than in the dogs where the resting flow was high No difference was noted between the Jorpes and the Boots' secretin. The flow response to pancreozymin showed the same features as seen for secretin a marked increase in some experiments, no significant increase in others (Fig 3 and 4)

The administration of secretin did not give any significant change in arterial blood pressure (ABP), while in most experiments pancreozymin led to an immediate lowering of ABP of 20—25 mm Hg lasting for 20 sec or more Similar reactions were seen after acetylcholine and histamine The decreased ABP resulted in a transient reduction of blood flow visualized by the flattening out of the desaturation curve (Fig 6)

The dissociation between secretory and blood flow response to hormonal stimulation is evident from Fig 5 In this experiment secretin and pancreozymin were given simultaneously A constant stimulation led to a steady secretion and mean blood flow was ca 38 ml/min 100 g As the hormone infusion was suddenly increased external secretion was trebled and quickly reached a new steady state A blood flow increase was also observed on average to 80 ml/min 100 g 5 min after the stimulatory change However the blood flow returned to its previous level in the course of 20—30 min despite a continuous high secretory rate

When the hormone stimulations were supplied suddenly during a desaturation run the slope of the curve almost immediately became steeper but only 3—5 min passed before the curve started to flatten out again (Fig 6) The change is due to flow variations and is caused by the hormones since no such responses occurred after saline infusion Acetylcholine and histamine gave no consistent effect on the pancreatic blood flow except for a short reduction corresponding to the change in ABP In contrast concommittant tracings from the gastric wall showed marked increases in the hydrogen desaturation curves after administration of these drugs while secretin and pancreozymin had no such effect

Recordings were also made of hormonal as well as vasoactive responses in 3 awake dogs The results were consistent with the observations made in Nembutal anesthesia

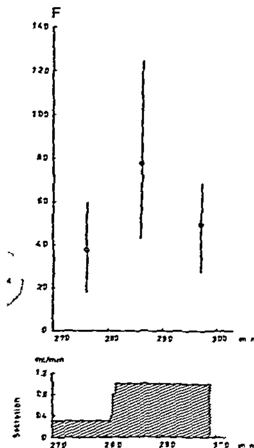


Fig. 5. Effect of a threefold increase in stimulation with a combination of secretin and pancreozymin on pancreatic blood flow (F ml/min 100 g) and external secretion (ml/min) in dog no. 9.

Discussion

In the present series the pancreatic blood flow was assessed by means of the hydrogen gas clearance technique of Aukland *et al.* 1964. This method seems to offer a valuable tool in the study of circulation of this organ since measurements can be performed with negligible trauma to the tissues; it can be applied both in the anesthetized and in the awake animal, and measurements can be repeated for long periods of time and under various conditions. Finally the method allows measurements in several organs simultaneously. Therefore integrated blood flow studies are possible as for instance in functionally related organs such as those in the digestive tract.

The best measurements of blood flow in the intact pancreas have previously been performed by means of K^{42} or Rb^{86} clearance techniques first described by Sapirstein (1958) and recently utilized by Delaney and Grim (1966) and Papp *et al.* (1965). Considerable divergencies appear between their findings. Whereas pancreatic blood flow in the resting state could be calculated by Sapirstein (1958) at

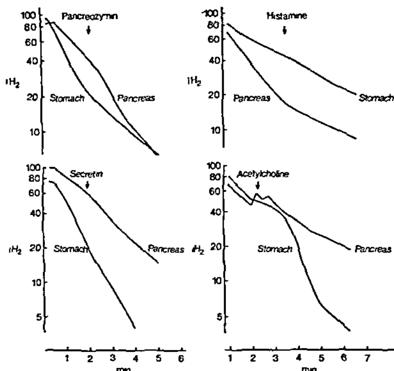


Fig 6 H_2 desaturation curves of pancreas and stomach wall in dog no 12 awake (left) and in *the same dog anesthetized (right) demonstrating the acute effect of pancreozymin, secretin* histamine and acetylcholine

100 ml/min 100 g tissue in the rat, the corresponding result was 61 ml according to Delaney and Grim in the dog (1966) These authors supposed the difference to be due to the size of the animals Another possible explanation is circulatory differences induced by anesthetics Delaney and Grim (1966) used pentobarbital in their dogs while Papp *et al* (1966) have reported resting flow at 75 ml/min 100 g with the use of chloralose anesthesia

Our results indicate that the pancreatic vasculature is sharing in the vasoconstrictive influence of Nembutal causing about 40 % reduction of the blood flow in the non stimulated pancreas Flow variations in our experiments might partly be caused by differences in the constrictory state of the vasculature individually and during each experiment No consistent pattern could be seen of local variations within the organ although such variations were noted (Fig 2) without obvious reason

Alterations in pancreatic blood perfusion have previously been studied by use of direct as well as indirect methods by which relative variations are obtainable Tankel and Hollander (1957) in their surveys of the literature up to that time, found no definite evidence of a correlation between blood flow and external secretion Even recent studies show discrepancies in assessing the effect of secretory stimulation and vasoactive substances Secretin is uniformly reported to have an increasing effect on the pancreatic blood flow although Lindgren (1958) found that the effect disappeared with repeated secretin injections

Whereas Delaney and Grim (1966) found no increasing effect of pancreozymin administration on pancreatic blood flow, a marked increase was reported by Barlow *et al* (1968) in their experiments with highly purified pancreozymin extracts in the anesthetized cat In the present study, no difference in the effect of secretin and pancreozymin was noted, both hormones causing a distinct but transient increase in the pancreatic blood perfusion The effect was of short duration compared to a rather persistent external secretion, which showed a definite dose response relationship The observed change of pancreatic blood flow seemed to depend on changes induced by the stimulant itself or its effect on pancreatic secretory tissue, since secretion could be maintained at a high rate even though the pancreatic blood perfusion was low or decreasing If the hormone affected the vasculature directly, by dilating arteries or arterioles, this influence predominantly attacks pancreas, since neighbouring organs like the stomach are left essentially unchanged

The flow effect is not due to a general vasoactivity since no effect was observed by the pancreatic hormones on the hydrogen clearance of the stomach On the other hand, no change was seen in pancreatic blood flow following administration of gastric secretagogues, *e g* histamine and acetylcholine, which caused an (instantaneously) increased hydrogen clearance from the stomach

A Preliminary report of this study was read at the annual meeting of the Norwegian Surgical Association Oct 28 1966

References

AUKLAND K B F BOWER and R N BERLINER Measurement of local blood flow with hydrogen gas *Circulat Res* 1964 *14* 164—187

AUKLAND K F KIIL J KJEKSHUS and G SEMB Local myocardial blood flow measured by hydrogen polarography distribution and effect of hypoxia. *Acta physiol scand* 1967 *70* 99—111

renal nerve stimulation on
d 1968 *72* 498—509
RD *Factors influencing pan*
s Ed W H Bain and A
169—481
e pancreatic blood
tion With observa
avec des segments

GAYET R and M GUILLAUMIE Les reactions vasomotorices du pancreas étudiees par la mesure des debiets sanguins *C B de la Soc de biol* 1930 *103* 1106 1110

GREGORY R A *Secretory Mechanisms of the Gastro-intestinal Tract* Edw Arnold (Publ) Ltd London 1962 p 144

HILTON P and M JONES Some observations on changes in the blood content of the cats pancreas during activity *J Physiol* (Lond) 1960 *150* 479—488

LINDGREN P, An improved method for drop-recording of arterial or venous blood flow *Acta physiol scand* 1958 *42* 5—11

M Effect of pancreatrophic agents on pan

M Pankreas bei humoraler An

pancreatic blood flow Arch int

ntitative study of pancreatic blood
51 524—528
on of ndicators *Amer J Phys*

local blood

TANKEL H and F HOLLANDER A flow A review *Gastroenterology* 1951 *32* 633—641

Acta physiol. scand. 1969. 76. 415—426

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Electron and Fluorescence Microscopical Studies on the Nucleus Caudatus Putamen of the Rat after Unilateral Lesions of Ascending Nigro-Neostriatal Dopamine Neurons

By

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Received 11 October 1968

Abstract

HOKFELT, T and U UNGERSTEDT *Electron and fluorescence microscopical studies on the nucleus caudatus putamen of the rat after unilateral lesions of ascending nigro-neostriatal dopamine neurons* Acta physiol scand 1969 76 415—426

[illegible]

the operation These results were in perfect agreement with the fluorescence microscopical findings, demonstrating a reduced accumulation of amines in the DA nerve terminals as well as reduced levels of endogenous amine at these time intervals after the lesion —Parallel to the reduction in number of boutons containing small granular vesicles boutons were observed showing obvious signs of the so called "dense" type of degeneration These boutons were however, comparatively rare and present above all in rats killed between two and four days after performing the lesion Further studies are necessary to establish whether they represent boutons belonging to degenerating DA nerve terminals or not It may be concluded that the present study gives ultrastructural evidence for the existence of an ascending nigro-neostriatal DA neuron system, although the degenerating pattern of these neurons is still unclear

There is good evidence for the existence of a large uncrossed nigro-neostriatal dopamine (DA) neuron system in the brain (Anden *et al* 1964 1965, Poirier and Sourkes 1965, Hornykiewicz 1966) The cell bodies localized mainly in the zona compacta of the substantia nigra (Dahlstrom and Fuxe 1964b, Anden *et al* 1965), send their axons to the nucleus caudatus putamen (NCP) where they form an extensive terminal plexus (Fuxe *et al* 1964 Fuxe 1965, Andén *et al* 1966c) So far it has not been possible to demonstrate this system with silver impregnation-degeneration studies, *e g* with Nauta technique (Cole *et al* 1964, Faull and Carman 1968), probably due to the fineness of the fibres (Fuxe *et al* 1964) However, the ascending nigro-neostriatal DA fibres has recently been mapped out in their entire course with fluorescence technique by studying the accumulation of transmitter sub-

TABLE I Effect of lesions of ascending nigro-neostriatal dopamine neurons on the nucleus caudatus putamen as revealed in the electron and fluorescence microscope

From the electron microscopic investigation on the nucleus caudatus putamen from the control and operated side the total number of all boutons, the boutons containing small granular vesicles ("positive"), as well as boutons showing obvious signs of degeneration have been recorded from 13 operated and 2 sham operated rats (see Material and methods) In addition the effect on the endogenous amine levels and the effect on the uptake and accumulation of exogenous amines in slices as revealed in the fluorescence microscope are summarized

Endogenous amine levels (the overall fluorescence of the nucleus caudatus putamen is recorded, since no single terminals can be observed)		Uptake and accumulation of exogenous amines in slices	
○○○	normal fluorescence intensity	▽▽▽	normal number of nerve terminals
○○	markedly reduced fluorescence intensity	▽▽	markedly reduced number of nerve terminals
○	no or very faint fluorescence	▽	no or very few nerve terminals

Time of survival (days)	Rat ident no	Electron microscopy							Fluorescence microscopy	
		Boutons total		Boutons positive		Boutons degenerated	Boutons % positive		Endogenous	Slices
		Lesion	Control	Lesion	Control		Lesion	Control		
1d	559	1674	1222	118	150	0	7 1	12 3	○○○	▽▽▽
	560	1626	1127	194	130	0	12 0	11 5	○○○	▽▽▽
							9 6*	11 9		
2d	515	1628	604	23	87	7	1 4	14 4	○	▽
	518	639	790	5	90	11	0 8	11 4	○	▽
							1 1	12 9		
3d	506	1033	756	43	96	0**	4 2	12 7	○○	▽▽
	514	1713	1151	32	134	8	1 9	11 6	○	▽
							3 1	12 2		
4d	586	802	301	25	32	1	3 1	10 6	○○	▽▽
	587	761	382	0	44	0**	0	11 5	○	▽
							1 6	11 1		
5d	571	679	427	21	53	0**	3 1	12 4	○○	▽▽
	576	656	294	10	28	0**	1 5	9 5	○○	▽▽
							2 3	11 0		
6d	563	560	388	7	39	0	1 3	10 1	○	▽
	565	733	588	9	63	0**	1 2	10 7	○	▽
							1 3	10 4		
12d	505	873	608	8	94	0**	0 9	15 5	○	▽
2d	634***	1524	—	169	—	0	12 7	—	○○○	▽▽▽
	637***	1035	—	131	—	0	11 1	—	○○○	▽▽▽
							11 9			

* Mean ** Single degenerated boutons observed *** Sham operated rats

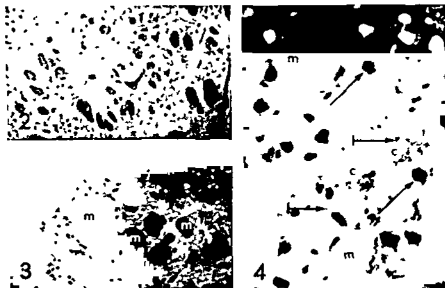


Fig 2 Slice of the NCP of rat incubated with α methyl NA (10 μg/ml) control side An intense diffuse fluorescence is seen The large dark spots are myelinated nerve bundles (m) Fluorescence micrograph ×75

Fig 3 4 Slice of the NCP of the same rat as in Fig 2 incubated with α methyl NA (10 μg/ml) operated side rat killed 4 days after the operation Only very few fluorescent nerve terminals are seen In Fig 4 single varicosities (↔) of the nerve terminals can be seen Note the large number of intensely fluorescent cell bodies of unknown nature (↗) m = myelinated nerve bundles c = non fluorescent nerve cell bodies Fluorescence micrographs ×75 and ×300 respectively

amine containing systems A close correlation was found between the extent to which the DA axons were lesioned and the disappearance of DA from the NCP as seen in the fluorescence microscope and the electron microscope (see below)

The NCP

Fluorescence microscopy No obvious difference in fluorescence intensity could be observed between the incubated slices from the operated and the control side of rats killed 1 day after the operation (Table I) In slices from rats killed 2 or more days after the operation a very marked decrease in the degree of accumulation of amine was observed on the operated side (Fig 2—4) In some of these rats *e g* rats no 587 (4 days) 565 (6 days) and 505 (12 days) almost no intraneuronal specific fluorescence at all could be observed after incubation with α methyl NA (10 μg/ml) *In these sections a large number of green fluorescent cell bodies of unknown nature could be observed* Fig 3 4)

The effect of the lesion on the endogenous DA levels was studied in that part of the NCP which was not sliced or incubated in α methyl NA (Table I) One day after the lesion there was no obvious difference in fluorescence intensity between the NCP of the operated and the normal side However after two days a gre



Fig 5a, b The NCP of rat killed 4 days after the operation operated (5a) and control (5b) side. Note the marked difference in fluorescence intensity between operated and control side Since almost no specific fluorescence at all is seen on the operated side the localization of the NCP is indicated by thin lines Fluorescence micrographs × 75

duction of fluorescence intensity was seen in the NCP of the operated side signifying a large decrease in endogenous DA levels (Fig 5a b) This decrease was apparent in all animals from the 2nd—12th day after the lesion The results on the endogenous DA levels were wholly consistent with the results obtained from the incubated slices Some variations however occurred between the animals A less extensive lesion was parallelled by a less obvious difference between the NCP of the operated and normal side

Electron microscopy The results obtained from the electron microscopic studies have been summarized in Table I They show that in slices of the NCP from the control side incubated with α methyl NA (10 μg/ml) boutons containing small granular vesicles constitute between 10 and 16 (mean 11 9) per cent of all boutons (Fig 6) In slices from the operated side about the same percentage was found only in rats killed 1 day after the operation No obvious morphological changes were found in those boutons which contained small granular vesicles and which were present in slices of the operated side 1 day after the lesion. In slices of rats with longer survival periods (2 to 12 days) a dramatic reduction was found (Fig 7 8) Thus only between 0 and 4 per cent of all boutons contained small granular vesicles in slices from these rats In addition in slices from the operated side of these animals boutons of the so called dense type (Colonnier 1964 Walberg 1964 Alksne *et al* 1966) were seen. *i e* the boutons shrink and darken and become engulfed in glial cytoplasm (Fig 7 8 They were most numerous in slices from rats killed two to four days after the operation In rats with longer survival periods only single degenerating boutons were seen. *It must however be pointed out that these degenerating boutons* also during day 2 to 3 were rare as compared to *e g* the number of boutons con



Fig 6 Slice of the NCP of rat incubated with a methyl NA (10 μg ml), control side A number of boutons containing small granular vesicles are seen (a) The majority of the bouton however, contain only agranular vesicles Electron micrograph, × 27 000

taining small granular vesicles in slices from the control side Further signs of degenerative processes were found in the cytoplasm of glial cell bodies where myelin-like material could be observed

In sham operated rats killed 2 and 3 days after the operation 11 9 per cent of all boutons contained small granular vesicles, *i e* about the same percentage as in the controls Furthermore, no boutons showing obvious signs of degeneration were found

Discussion

Strong evidence now exists that small granular vesicles are storage sites for monoamines both *in the peripheral* (de Robertis and Pellegrino de Iraldi 1961a b Wolfe *et al* 1962 Richardson 1964 Bloom and Barrnett 1966 van Orden *et al* 1966, 1967a, b Hokfelt 1966 1967a) and the central nervous system (Hokfelt 1967b 1968 Hokfelt and Jonsson 1968) and that small granular vesicles are characteristic of boutons belonging to monoamine neuron systems Since biochemical studies ha revealed that DA concentrations are very high in the NCP (Bertler and Rosen



Figs. 7–8. Slices of the NCF of rat incubated with α-methyl NA (10 μg/ml), operated side, rat killed 2 days after the operation. In Fig. 7 one degenerating bouton (d) is seen surrounded by glial cytoplasm. One bouton (a) contains small granular vesicles. In Fig. 8 two boutons showing signs of degeneration are seen (d), both surrounded by the cytoplasm (g) of the same glial cell. Electron micrographs ×30 000 and ×38 000 respectively.

1959, Carlsson 1959) and that both NA and 5 HT concentrations are low (Poirier and Sourkes 1965, Sourkes and Poirier 1965, Andén, unpublished data), it may be assumed that the majority of boutons containing small granular vesicles in the NCP, indeed belong to DA nerve terminals (Hökfelt 1968), especially in view of the fact that fluorescence histochemical studies have demonstrated huge numbers of DA nerve terminals in the NCP (Andén *et al* 1965, Andén *et al* 1966c)

The results in the present study show that a unilateral lesion in a well defined area medial to the crus cerebri causes distinct changes in the ipsilateral NCP, whereas no obvious changes of the same type are found in the control (unoperated) side Thus, in thin brain slices of the NCP from the operated side only a very small percentage (0—4 per cent) of the total number of boutons contains small granular vesicles after incubation in *a* methyl NA (10 μg/ml) from day 2 and onwards This is in contrast to slices from the control side, where boutons containing small granular vesicles constitute between 10 and 16 per cent (mean 11 9 per cent) of all boutons This figure is somewhat lower than that obtained in a previous combined fluorescence and electron microscopic study (Hökfelt 1968), where about 16 per cent of all boutons contained small granular vesicles after incubation in an *a* methyl NA (1 μg/ml) containing medium. This difference is probably explained by a combined effect of regional differences and the small areas investigated in the electron microscope

The fluorescence microscopic results are in agreement with the electron microscopic findings No reduction in the fluorescence of endogenous amine was seen one day after the lesion whereas a strong reduction occurred after 2 days The slices showed a clearly reduced uptake of exogenous amine from day 2 and onwards

The decrease in boutons containing small granular vesicles is very marked, but there is still a varying number of such boutons present in almost all sections investigated This may be explained in several ways Firstly the lesion destroys varying numbers of DA axons A close correlation existed between the extent of the lesion and the number of boutons containing small granular vesicles Secondly, the remaining boutons containing small granular vesicles belong to NA and/or 5 HT neuron systems ascending close to the DA fibers at the level of the lesion (Andén *et al* 1966a) Thirdly there exists regional differences in the degree of degeneration within the NCP Due to the comparatively small areas investigated in the electron microscope such differences may not be observed and so far no attempt has been made to map out these possible differences in regional degeneration patterns

Not only did the number of boutons containing small granular vesicles decrease but there also appeared a special type of bouton in the NCP on the operated side These boutons showed typical signs of degeneration of the dark type *i e* aggregation of synaptic vesicles darkening and shrinking of the bouton which was engulfed in glial cytoplasm (Colonnier 1964 Alksne *et al* 1966) It is tempting to assume that these boutons belong to degenerating DA nerve terminals However, doubts can be cast to this assumption *e g* the number of degenerating comparatively low or about one per cent at the most Thus there is no

respondence between the loss of boutons containing small granular vesicles and boutons showing degenerative changes. This difference however may at least partly be explained by the possibility that degenerating boutons may be comparatively quickly removed and broken down by the glial cells. In this connexion it may be pointed out that in this study only boutons with very obvious signs of degeneration have been counted. *This is partly due to the fact that the morphology of brain slices* as processed in the present study is clearly inferior to that obtained after *e.g.* perfusion with glutaraldehyde, mainly due to the incubation procedure (Hökfelt 1968). Thus early changes in degenerating boutons are difficult to determine with certainty.

It must also be kept in mind that the degenerating boutons observed may belong to axons ascending close to the DA fibers. If thus DA nerve terminals do not show a degeneration pattern of the dense type it may be speculated that with the present technique boutons belonging to DA neurons at least during the first six days may appear as normal boutons with agranular vesicles. Further studies with longer survival times may solve this problem.

Electron microscopic and fluorescence microscopic results obtained in this study show that after destruction of the DA fibres to the NCP the majority of the boutons of the DA nerve terminals lose their ability to take up amines at the level of the cell membrane and to accumulate these amines at the granular storage sites as well as to maintain endogenous amine levels.

Previous studies on degenerating sympathetic adrenergic neurons have shown loss of or a decreased ability to take up and retain exogenous NA (Malmfors and Sachs 1965; Smith *et al.* 1966). Furthermore there is a reduction in the *in vivo* incorporation of H^3 dopa in central CA neurons after lesions in the medial forebrain bundle (Moore *et al.* 1965). Further signs of degeneration in the NCP after damage to the ascending DA fibres are decreases in tyrosine hydroxylase (Goldstein *et al.* 1966) and dopa-decarboxylase activity (Andén *et al.* 1966a).

Several investigations have postulated a nigro-neostriatal DA system (see above). In the present study severed DA axons could be traced back to cell bodies in the substantia nigra due to intraneuronal accumulation of fluorescent material and the DA nerve terminals in the NCP showed almost complete amine depletion. Furthermore large lesions caudal to the substantia nigra have shown that probably no DA fibres ascend from the pons and the medulla oblongata (Andén *et al.* 1966b). There is thus little doubt that the lesion of this study damaged DA fibres originating from cell bodies in the substantia nigra. In the electron microscope these lesions caused a reduction of a special type of bouton in the NCP previously identified as belonging to DA terminals. This fact give ultrastructural evidence for the existence of a nigro-neostriatal DA system.

This study was supported by grants from the Swedish Medical Research Council (B69-14X-715-04A) and by grants from "Stiftelsen Therese och Johan Anderssons minne", "Svenska Livförsäkringsbolags nämnd för medicinsk forskning", "Magnus Bergvalls Stiftelse" and "Ollie och Elof Ericssons Stiftelse".

The skilful technical assistance of Mrs. Waldtraut Hiort and Miss Kerstin Sterner is gratefully acknowledged.

References

ALKSNE, J F, T W BLACKSTAD, F WALBERG and L E WHITE, JR., Electron microscopy of axon degeneration A valuable tool in experimental neuroanatomy *Ergebn Anat Entwickl-Gesch* 1966 *39* 1—32

ANDEN, N E, A CARLSSON, A DAHLSTRÖM, K FUXE, N-A HILLARP and K LARSSON, Demonstration and mapping out of nigro-neostriatal dopamine neurons *Life Sci* 1964 *3* 523—530

ANDEN, N-E, A DAHLSTRÖM, K FUXE and K. LARSSON, *Further evidence for the presence* of nigro-neostriatal dopamine neurons in the rat *Amer J Anat* 1965 *116* 329—333

ANDEN, N-E, A DAHLSTROM, K FUXE, K LARSSON, L OLSON and U UNGERSTEDT, Ascending monoamine neurons to the telencephalon and diencephalon *Acta physiol scand* 1966a *67* 313—326

ANDEN, N-E, A DAHLSTRÖM, K FUXE, L OLSON and U UNGERSTEDT, Ascending noradrenaline neurons from the pons and the medulla oblongata *Experientia* (Basel) 1966b *22* 44

ANDEN, N-E, K FUXE, B HAMBERGER and T HÖKFELT, A quantitative study on the nigro-neostriatal dopamine neuron system in the rat *Acta physiol scand* 1966c *67* 306—312

BERTLER, A and E ROSENGREN, Occurrence and distribution of dopamine in brain and other tissues *Experientia* (Basel) 1959 *15* 10—11

BLOO [illegible] in vesicles of au [illegible]

CARL [illegible] —304

CARL [illegible] histochemical studies on the effects of imipramine like drugs and (+) amphetamine on central and peripheral catecholamine neurons *Acta physiol scand* 1966 *67* 481—497

CARLSSON, A, N-A HILLARP and B WALDECK, Analysis of the Mg^{++}-ATP dependent storage mechanism in the amine granules of the adrenal medulla *Acta physiol scand* 1963 *59* Suppl 215 1—38

CARLSSON, A and B WALDECK Mechanisms of amine transport in the cell membranes of adrenergic nerves *Acta pharmacol* (Kbh) 1965 *22* 293—300

COLE, M, W J H NAUTA and W R MEHLER The ascending efferent projections of the substantia nigra *Trans Amer neurol Ass* 1964 *89* 74—78

COLONNIER, M, Experimental degeneration in the cerebral cortex *J Anat* (Lond) 1964 *98* 47—53

COLONNIER M and E G GRAY, Degeneration in the cerebral cortex. *Electron Microscopy* 1962 Ed S S Breese Jr Academic Press N Y 2 L-3

CORRODI, H and G JONSSON, The formaldehyde fluorescence method for the histochemical demonstration of biogenic monoamines A review on the methodology *J Histochem Cytochem* 1967 *15* 65—78

DAHLSTRÖM A, *The intraneuronal distribution of noradrenaline and the transport and life-span of amine storage granules in the sympathetic adrenergic neuron* M D Thesis Stockholm 1966

DAHLSTRÖM A. and K FUXE A method for the demonstration of monoamine containing nerve fibres in the central nervous system *Acta physiol scand* 1964a *60* 293—295

DAHLSTRÖM, A and K FUXE Evidence for the existence of monoamine neurons in the central nervous system. I Demonstration of monoamines in the cell bodies of brain stem neurons *Acta physiol scand* 1964b *62* Suppl 232 1—55

DE ROBERTIS, E and A PELLEGRINO DE IRALDI Plurivesicular secretory processes and nerve endings in the pineal gland *J biophys biochem Cytol* 1961a. *10* 361—372

DE ROBERTIS E and A PELLIGRINO DE IRALDI, A plurivesicular component in adrenergic nerve endings *Anat Rec* 1961b *139* 299

EULER U S VON *and* F LISHAJKO *Catecholamine release and uptake in isolated adrenergic* nerve granules *Acta physiol scand* 1963a. *57* 468—480

EULER [illegible] and up [illegible]

FALCK [illegible] lated [illegible] 354

FAULL, R L M and J B CARMAN Ascending projections of the substantia nigra in the rat *J comp Neurol* 1968 *132* 73—92

FUXE, K, Evidence for the existence of monoamine neurons in the central nervous system IV Distribution of monoamine nerve terminals in the central nervous system *Acta physiol scand* 1965 *64* Suppl 247 37—84

FUXE K and N A HILLARP Uptake of l-dopa and noradrenaline by central catecholamine neurons *Life Sci* 1964 *3* 1403—1406

FUXE K., T HOKFELT and O NILSSON, Observations on the cellular localization of dopamine in the caudate nucleus of the rat *Z Zellforsch* 1964 *63* 701—706

FUXE K. and G JONSSON A modification of the histochemical fluorescence method for the improved localization of 5-HT *Histochemie* 1967 *11* 161—166

GOLDSTEIN M B B ANAGNOSTE W S OWEN and A F BATTISTA The effects of ventromedial tegmental lesions on the biosynthesis of catecholamines in the striatum *Life Sci* 1966 *5* 2171—2176

GRILLO M and S L PALAY Granule-containing vesicles in the autonomic nervous system *Electron Microscopy* 1962 Ed. S S Breese Jr Academic Press N Y *2* U-1

HAMBERGER B Reserpine resistant uptake of catecholamines in isolated tissues of the rat *Acta physiol scand* 1967 *295* 1—56

HORNYKIEWICZ O Metabolism of brain dopamine in human parkinsonism neurochemical and clinical aspects *Biochemistry and pharmacology of basal ganglia* 1966 Ed E Costa L J Cote and M D Yahr Raven Press-Hewlett, N Y *171—181*

HOKFELT, T The effect of reserpine on the intraneuronal vesicles of the rat vas deferens *Experientia* (Basel 1966 *22* 56—57

HOKFELT T Ultrastructural studies on adrenergic nerve terminals in the albino rat iris after pharmacological and experimental treatment. *Acta physiol scand* 1967a. *69* 125—126

HOKFELT T On the ultrastructural localization of noradrenaline in the central nervous system of the rat. *Z Zellforsch* 1967b *79* 110—117

HOKFELT T *In vitro* studies on central and peripheral monoamine neurons at the ultrastructural level *Z Zellforsch* 1968 *91* 1—74

HOKFELT T and G JONSSON Studies on reaction and binding of monoamines after fixation and processing for electron microscopy with special reference to fixation with potassium permanganate *Histochemie* 1968 *16* 45—67

LUFT J H Improvements in epoxy resin embedding methods *J biophys biochem Cytol* 1961 *9* 409—414

MALMFORS T Studies on adrenergic nerves The use of rat and mouse iris for direct observations on their physiology and pharmacology at cellular and subcellular levels *Acta physiol scand* 1965 *64* Suppl 248 1—93

MALMFORS T and CH SACHS Direct studies on the disappearance of the transmitter and the changes in the uptake storage mechanisms of degenerating nerves *Acta physiol scand* 1965 *64* 211—223

MC ILWAIN H and R. RODNIGHT *Practical neurochemistry* 1962 J A Churchill London

MOORE R. Y R L MARGOLIS and A HELLER, Incorporation of DOPA H^3 into brain catecholamines following hypothalamic lesions in the cat *Fed Proc* 1965 *24* 195

ORDEN L S VAN K G BENSCH and N J GIARMAN Histochemical and functional relationships of catecholamines in adrenergic nerve endings II Extravesicular norepinephrine *J Pharmacol exp Ther* 1967a *155* 428—439

ORDEN L S VAN K G BENSCH S Z LANGER and U TRENDELENBURG Histochemical and fine structural aspects of the onset of denervation supersensitivity in the nictating membrane of the spinal cat *J Pharmacol exp Ther* 1967b *157* 274—283

ORDEN L S VAN F E BLOOM R J BARRNETT and N J GIARMAN Histochemical and functional relationships of catecholamines in adrenergic nerve endings I Participation of granular vesicles *J Pharmacol exp Ther* 1966 *154* 185—199

POIRIER L J and T L SOURKES Influence of the substantia nigra on the catecholamine content of the striatum *Brain* 1965 *88* 181 192

REYNOLDS E S The use of lead citrate at high pH as an electron-opaque stain in electron microscopy *J Cell Biol* 1963 *17* 208—212

RICHARDSON K C The fine structure of the albino rabbit iris with special reference to the identification of adrenergic and cholinergic nerves and nerve endings in its intrinsic muscles *Amer J Anat* 1964 *114* 173—206

RICHARDSON K C Electron microscopic identification of autonomic nerve endings *Nature* (Lond.) 1966 *210* 756

SMITH C. B U TRENDELENBURG S Z LANGER and T H TSAI The relation of norepinephrine H^3 to the norepinephrine content of the nictating membrane of the spinal cat during development of denervation supersensitivity *J Pharmacol exp Ther* 1966 *151* 87—94

SOURKES T L and L J POIRIER Influence of the substantia nigra on the concentration of 5-hydroxytryptamine and dopamine of the striatum *Nature* Lond 1965 *207* 202—203

WALBERG, F The early changes in degenerating boutons and the problem of argyrophilia. Light and electron microscopic observations *J comp Neurol* 1964 *122* 113—137

WOLFE, D E., J AXELROD L T POTTER and K C RICHARDSON Localizing tritiated norepinephrine in sympathetic axons by electron microscope autoradiography *Science* 1962 *138* 440—442.

Acta physiol. scand. 1969. 76. 427—440

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Distribution of Noradrenaline Storing Particles in Peripheral Adrenergic Neurons as Revealed by Electron Microscopy

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Received 11 October 1968

Abstract

HÖKFELT, T. *Distribution of noradrenaline storing particles in peripheral adrenergic neurons as revealed by electron microscopy* Acta physiol scand 1969 *76* 427—440

The cell bodies, the axons and the terminal parts of peripheral sympathetic neurons originating in the superior cervical ganglion and terminating in the dilator muscle of the iris were investigated in the rat by electron microscopy using potassium permanganate as a fixative In all parts of the neuron granular vesicles were found They were most numerous in the axonal [illegible] small granular vesicles were in majority, constituting about [illegible] enlargements in the dilator muscle of the iris The number of vesicles in the axonal enlargements varied widely from 45 to 852 per enlargement In all probability the small granular vesicles are the main storage site of noradrenaline in the peripheral adrenergic neurons However, also the large ones probably contain an amine or at least are involved in the amine metabolism

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In a series of papers Euler (1946a b c 1948, 1950) presented strong evidence that noradrenaline (NA) is the predominant catecholamine (CA) in peripheral tissues and adrenergic nerves Subsequent studies indicated that much higher concentrations of NA were present in the nerve terminals than in the preterminal axon and the cell body of the neurons (Rexed and Euler 1951 Euler 1957) Furthermore, fundamental data on the subcellular localization of the intraneuronal NA was presented by Euler and Hillarp (1956) by demonstrating particle bound NA in homogenates from bovine splenic nerves Thus the first evidence had been obtained for the existence of NA storage granules in nerves similar to those previously demonstrated in adrenal medullary cells (Blaschko and Welch 1953 Hillarp *et al* 1953)

With the help of the histochemical formaldehyde fluorescence method of Falck and Hillarp (Falck *et al* 1962 Hillarp *et al* 1966) these findings were definitely established and it was even possible to achieve quantitative data on the intraneuronal distribution of NA in single neurons (Norberg and Hamberger 1964, Dal[illegible] 1966)

and showed the same features as those found in the terminal parts of the neurons both with regard to size and morphological features The small granular vesicles were found in large amounts in some cell bodies whereas they were rather sparse in others They were found in high numbers in the peripheral parts (Fig 1) of the cell body especially where the cell processes emanate from the cell bodies (Fig 1) Here often clusters of small granular vesicles could be seen (Fig 1, 3) However also along the cell membrane and in connection with the Golgi apparatus small granular vesicles were found (Fig 2) It must however, be pointed out that the size of the granular vesicles in the neighbourhood of the Golgi apparatus was less uniform than *e g* in the peripheral parts of the cell body This may indicate that granular vesicles in this region are involved in a growth process and possibly formed in association to the Golgi apparatus In addition also small granular vesicles were found often in high numbers in cell processes probably both in axons and dendrites (Fig 1 4 5) Thus small granular vesicles could sometimes be seen lying in processes which were in synaptic contact with presynaptic presumably cholinergic nerve endings (Fig 5) *The latter are recognized by the fact that all vesicles both the small and the large ones* are agranular or have an interior of only a very low density It is however obvious that only serial sectioning can reveal the true structural relationships in the superior cervical ganglion (*cf* Elfvin 1963a b)

Large granular vesicles could be seen together with the small granular vesicles in the same parts of the cell bodies and the processes where small granular vesicles were observed although mostly only few were present They thus are few as compared to the small ones

This first type of cell bodies may correspond to NA containing cell bodies previously demonstrated in this ganglion (Hamberger *et al* 1963)

In the *second type* of cell bodies neither small nor large granular vesicles were observed Only vesicles of the agranular type of varying size were seen mostly in the neighbourhood of the Golgi apparatus These cell bodies may correspond to cell bodies with high acetylcholinesterase activity described by Eranko and Harkonen (1964) in this ganglion

A special kind of cell bodies constituted the *third group* These cell bodies were comparatively rare and mostly lying in small groups They were smaller than the majority of the cell bodies and large numbers of granular vesicles with a diameter of about 800—1200 Å were found throughout the cytoplasm In addition empty vesicles of about 2000 to 3000 Å size were seen in these cell bodies These cell bodies may correspond to so called small intensely fluorescent (SIF) cells previously demonstrated in fluorescence microscopic studies (Eranko and Harkonen 1963 Norberg *et al* 1966) and also described at the ultrastructural level (Siegrist *et al* 1966 Elfvin 1967) They are believed to contain a primary CA (Norberg *et al* 1966)

The internal carotid nerve trunk

The majority of the axons of the internal carotid nerve trunk were thin unmyelinated axons with a diameter of about 0 5 to 1 0 μ surrounded by Schwann cell cytoplasm

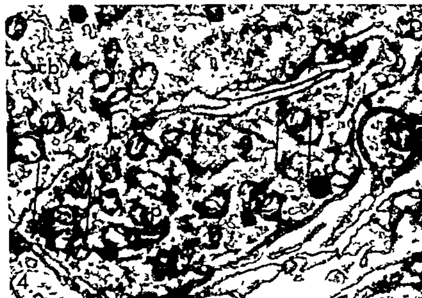




Fig. 4. Superior cervical ganglion of rat. A cell process (p), probably a dendrite, is seen in close contact with a cell body (cb). Note the high number of granular vesicles in the cell process (↗). × 17 000

Fig. 5. Superior cervical ganglion of rat. A preganglionic, probably cholinergic nerve ending (c) makes synaptic contact (▶) with a process (p) of a cell body. Note the presence of granular vesicles (↗) in the cell process. × 59 000



Fig 6 Internal carotid nerve trunk of rat. Only a few granular vesicles (↗) are seen in the unmyelinated axons (a) 29 000

Fig 7 Internal carotid nerve trunk of rat. A single unmyelinated axon (a) surrounded by a comparatively wide extracellular space (e). An aggregation of mainly small granular vesicles is seen in the axonal cytoplasm (▶) 37 500

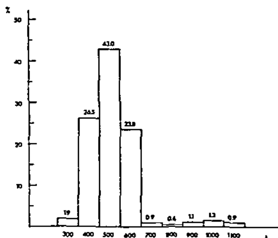


Fig 8 Distribution of diameters of granular vesicles in axonal enlargements in the dilator muscle of rat iris About 500 vesicles were measured Two types of vesicles seem to be present, distinguished by their size Considering 750 Å as a border line between the two types they will have a mean diameter of 495 Å and 967 Å respectively

Single myelinated axons were seen only occasionally Within these axons both small and large granular vesicles were present although in much lower concentrations than in the nerve terminals in the iris and in many cell processes in the superior cervical ganglion (Fig 6, 7) However, occasionally clusters of mainly small granular vesicles could be seen in single axons (Fig 7) The relative proportions between small and large granular vesicles in the axons were difficult to estimate properly, since careful serial sectioning was not performed However it seemed as if the proportion of large granular vesicles was relatively higher in the axons than in the cell bodies and the nerve terminals

Within the axons tubular structures and varying numbers of agranular vesicles of different size were seen Sometimes the vesicles lay close together along a line parallel to the long axis of the axon Mitochondria were frequently seen within the axons (Fig 6)

The dilator muscle of the iris

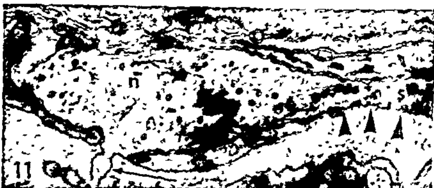
The axons in the dilator muscle of the rat iris were usually lying in bundles where a varying number of axons (up to 11) were surrounded by a Schwann cell However single axons completely free of Schwann cells were also seen

At least two types of axons were seen distinguished by the type of their intraneuronal vesicles (Fig 9) Thus with the present fixation technique one type probably the adrenergic axons contained almost exclusively vesicles with an electron dense interior so called granular vesicles This dense interior either consisted of one single dense core of a varying size or of two or more small dense cores (Fig 9) Often a few vesicles without a dense core were seen in the same axons together with the granular vesicles

The diameter of the vesicles in the adrenergic axons has been given in Fig 8 Th results indicate the presence of two types of vesicles with a diameter of about and 1000 Å respectively corresponding to the small and large granular v







[illegible] seen. One (n) contains mainly [illegible] re that in both [illegible] are seen (↗↦) [illegible] (↦) × 36 500

Fig 10 Dilator muscle of rat iris Axonal enlargement (n) containing granular vesicles with a triple layered membrane The enlargement is partly free of Schwann cell cytoplasm (▶) × 58 000

Fig 11 Dilator muscle of rat iris. An axonal enlargement (n and part of a thin axonal segment (s) is seen. Only few granular vesicles are present in the enlargement while comparatively many granular vesicles are seen in the thin segment Note that the thin segment is free of Schwann cell cytoplasm (▶) and the extrusion of the axon (↗) × 27,000

TABLE I The number of granular vesicles in axonal enlargements in the dilator muscle of the rat iris as revealed in serial sections

14 axonal enlargements containing granular vesicles have been followed in serial sections In every section the number of vesicles were counted and summarized for each enlargement Vesicles with a diameter above 750 Å (large vesicles) were counted separately (see Fig 1) Correction for double counting was performed according to Billingsley and Ransom (1918—19)

Axonal enlargement	Vesicles		Vesicles after correction	
	Small	Large	Small	Large
1	72	0	45	0
2	132	2	82	1
3	214	11	133	5
4	361	12	224	5
5	399	7	247	3
6	404	1	251	1
7	444	8	275	4
8	472	13	293	6
9	484	9	300	4
10	637	14	395	7
11	658	18	407	9
12	749	25	464	12
13	773	13	479	6
14	1374	34	852	17
Mean	512	12	318	6

classified by Grillo and Palay (1962) If the vesicles are divided into two groups with 750 Å as a border line the mean diameter of the two groups can be calculated to be 495 Å and 967 Å respectively

The second type of axon probably the cholinergic axons contained vesicles only of the agranular type i e their interior was of a very low electron density (Fig 9) However also in these axons the vesicles may tentatively be divided in small and large ones the latter being comparatively rare

The intraneuronal distribution of the granular vesicles in the adrenergic axons in the dilator muscle of the rat iris has been studied partly with the help of serial sections The granular vesicles seemed to be present in most parts of the nerve terminals although only few vesicles were present in axons and axon bundles running in front of the vessels in the iris Occasionally clusters of mainly small granular vesicles were found in single axons

Axons running between blood vessels and approaching the smooth muscle layer often enlarged at intervals and within these enlargements usually high numbers of granular vesicles were present (Fig 9 10 Here the axon often was partly or e completely free of Schwann cell cytoplasm (Fig 10) Fourteen such enlar

have been followed in serial sections and the number of vesicles in each section counted As shown in Table I the number of vesicles in these enlargements varied widely, from 45 to 862 (mean 318) after correction for double counting according to Billingsley and Ransom (1918—1919) It has, however, to be pointed out that 862 by no means represent an upper limit Thus, axonal enlargements of larger dimensions containing very high numbers of granular vesicles were seen, although they could not be followed in series of sections All vesicles with a diameter above 750 Å were counted separately and found to constitute about 1 8 % of all vesicles These large granular vesicles were often found either in the thin segments of the axons at the ends of the axonal enlargements In addition to the granular vesicles always a number of mitochondria was found within each axonal enlargement This finding may be of interest in connexion with the possible intraneuronal localization of monoamine oxidase (see Stjarne 1966 Jarrott and Iversen 1968)

It must be pointed out that many exceptions to the above mentioned description of the distribution and feature of the granular vesicles were found Thus granular vesicles were present also in the thin segments of the axons (Fig 11) and sometimes enlarged parts of axons contained almost no granular vesicles at all Furthermore axonal enlargements containing many granular vesicles were often localized far away from the effector cells i e the smooth muscle cells and sometimes surrounded by Schwann cell cytoplasm On the other hand certain thin segments of the axon (Fig 11 and axonal enlargements with only few granular vesicles could be seen partly free of Schwann cell cytoplasm

Discussion

At least two types of granular vesicles exist in peripheral autonomic neurons in the rat according to the classification of Grillo and Palay (1962) so-called small and large granular vesicles In the present study measurements of the diameter of granular vesicles indeed gave evidence for the existence of two types with mean diameters of 495 Å and 967 Å respectively These results are in good agreement e g with those of Euler and Swanbeck (1964) and Machado (1967)

The proportions of small and large granular vesicles seem to vary considerably in different neuron systems (see e g Hokfelt 1968) although the small ones almost always are the predominant type Thus in the present study the large ones constituted 1 8 per cent of all vesicles This figure may be compared with the 2 3 per cent found by Machado (1967) in nerve terminals in the rat pineal gland and 4 per cent found by Farrell (1968 in nerve terminals in the rat vas deferens

Almost complete agreement exists that the small granular vesicles are storage sites of NA in peripheral adrenergic neurons (for references see Hokfelt 1968) Recent evidence furthermore indicates that also in the central nervous system monoamines are mainly stored in small granular vesicles (Hokfelt 1967b 1968)

The role of the large granular vesicles has been more controversial The reasons for this may at least partly be that especially when using glutaraldehyde-osmium

tetroxide fixation large granular vesicles can be seen both in monoamine neurons and in non monoaminergic neurons (Coupland 1965, Fuxe *et al* 1965 1966 Clementi *et al* 1966, Grillo 1966) Furthermore, in several studies no obvious changes in number and appearance of these vesicles were found after various pharmacological treatments known to change amine levels (Fuxe *et al* 1965, 1966, Hokfelt 1966a, Bloom and Aghajanian 1968, Farrell 1968) In contrast to these results clear changes in the density of the large granular vesicles were obtained in recent pharmacological experiments using $KMnO_4$ as a fixative (Hokfelt 1968, and unpublished data) This may depend on the fact that the dense core observed after $KMnO_4$ fixation reflects a reaction between the fixative and the amine (Hökfelt and Jonsson 1968 *cf* Wood 1966), while *e g* after glutaraldehyde fixation the dense core rather reflects a reaction between the fixative and the matrix of the vesicles Evidence for the localization of amines in the large granular vesicles has recently also been obtained by Tranzer and Thoenen (1968) in histochemical studies and by using injections of 5 hydroxy dopamine Furthermore, in studies on rat heart based on linear sucrose density centrifugation it was recently possible to demonstrate the presence of two fractions containing 'light and 'heavy particles respectively (Roth *et al* 1968), both for which stored NA

In the present study small and large granular vesicles were found to be present in all parts of the adrenergic neuron and usually the highest number was found in the terminal parts whereas the lowest numbers were found in the axons However, in some adrenergic cell bodies high numbers of granular vesicles were found especially in the peripheral parts of the cytoplasm Also in single axons clusters of granular vesicles were seen These data are almost in complete agreement with fluorescence microscopical studies on the distribution of NA in peripheral adrenergic neurons (Norberg and Hamberger 1964 Malmfors 1965 Dahlstrom 1966) Earlier electron microscopic studies have revealed granular vesicles in cell bodies (Cravioto 1962 Taxi 1965 Grillo 1966) in axons (Elfvin 1961 Kapeller and Mayor 1967) and in nerve terminals (see *e g* Richardson 1964) of sympathetic adrenergic neurons However with the present fixation technique it has been shown that practically all vesicles in the adrenergic ground plexus contain a dense core and that both types of granular vesicles exist also in the cell bodies and in the axons of adrenergic neurons with considerable numbers of small granular vesicles in the cell bodies

The results in the present study may be correlated to problems concerning site of synthesis and intraneuronal transport of amine storage particles The presence of granular vesicles in all parts of the neuron may be considered to favour the view that the granules are manufactured in the cell body and transported down to the terminal parts of the neuron (see Dahlstrom 1966 Kapeller and Mayor 1967) Thus granular vesicles of both the small and large type were found in the neighbourhood of the Golgi apparatus This suggests that the Golgi apparatus is the site of synthesis of granular vesicles (Fuxe *et al* 1966 However since vesicles in this region may be in a state of growth it is difficult to state definitely that small granular vesicles indeed are synthesized in the Golgi apparatus Thus the present data do not

the possibility that especially small granular vesicles may be produced locally in various parts of the neuron. Recent studies on regenerating axons have even been interpreted to indicate formation of large granular vesicles in axons (Pellegrino de Iraldi and de Robertis 1968)

This study was supported by grants from the Karolinska Institutet (Reservationsanslaget), by grants from the Swedish Medical Research Council (B69-14X-715 04A) and by grants from "*Stiftelsen Therese och Johan Anderssons Minne*", "*Svenska Livförsäkringsbolags nämnd för medicinsk forskning*", "Magnus Bergvalls Stiftelse" and "Ollie och Elof Ericssons Stiftelse"

The skilful technical assistance of Mrs Waldtraut Hiort is gratefully acknowledged

References

BILLINGSLEY, P R and S W RANSOM, On the number of nerve cells in the ganglion cervicale superius and of the nerve fibres in the cephalic end of the truncus sympaticus in the cat and on the *numerical relations of preganglionic and postganglionic neurons* *J comp Neurol* 1918—1919 *29* 359—366

BLASCHKO H and A D WELCH, Localization of adrenaline in cytoplasmic particles of the bovine adrenal medulla. *Naunyn-Schmiedeberg's Arch exp Path Pharmak* 1953 *219* 17—22

BLOOM, F E and G K AGHAJANIAN, An electron microscopic analysis of large granular synaptic vesicles of the brain in relation to monoamine content *J Pharmacol exp Ther* 1968 *159* 261—273

[illegible], F P MANTEGAZZA and M BOTTURI A pharmacologic and morphologic study on *the nature of the dense-core granules present in the presynaptic endings of sympathetic ganglia* *Int J Neuropharmacol* 1966 *5* 281—285

COUPLAND R E Electron microscopic observations on the structure of the rat adrenal medulla II Normal innervation *J Anat* (Lond) 1965 *99* 255—272

CRAVIOTO H Electronenmikroskopische Untersuchungen am synaptischen Nervensystem des Menschen I Nervenzellen *Z Zellforsch* 1962 *58* 312—330

DAHLSTRÖM A *The intraneuronal distribution of noradrenaline and the transport and life span of amine storage granules in the sympathetic adrenergic neuron* M D Thesis Stockholm 1966

ELFVIN L-G The ultrastructure of unmyelinated fibers in the splenic nerve of the cat *J Ultrastruct Res* 1958 *1* 428—454

ELFVIN L G Electron microscopic investigation of filamentous structures in unmyelinated fibers of cat splenic nerve *J Ultrastruct Res* 1961 *5* 51—64

ELFVIN, L G The ultrastructure of the superior sympathetic ganglion of the cat I The structure of the ganglion cell processes as studied by serial sections *J Ultrastruct Res* 1963a. *8* 403—440

ELFVIN, L G The ultrastructure of the superior cervical sympathetic ganglion of the cat II The structure of the preganglionic end fibers and the synapses as studied by serial sections *J Ultrastruct Res* 1963b *8* 441—476

ELFVIN L-G A new granule-containing nerve cell in the inferior mesenteric ganglion of the rabbit *J Ultrastruct Res* 1968 *22* 37—44

EULER U S VON The presence of a substance with sympathin-E properties in spleen extracts *Acta physiol scand* 1946a. *11* 168—186

EULER, U S VON The presence of a sympathomimetic substance in extracts of mammalian heart *J Physiol* (Lond 1946b *105* 38—44

EULER, U S VON A specific sympathomimetic ergone in adrenergic nerve fibres (sympathin [illegible] *physiol scand* 1946c *12* 73—97

E[illegible] *nervous system* 1957 Ed D Richter

E[illegible] the presence of noradrenaline in submicroscopic structures of adrenergic axons *Nature* (Lond 1956 *177* 44 45

EULER, U S VON and G SWANBECK Some morphological features of catecholamine storing nerve vesicles. *Acta physiol scand* 1964 *62* 487—488

ERÄNKÖ, O and M HÄRKÖNEN Histochemical demonstration of fluorogenic amines in the [illegible] *Acta physiol scand* 1963 *58* 285 286

[illegible] in sympathetic ganglion [illegible]

[illegible] of catecholamines and [illegible]

related compounds condensed with formaldehyde *J Histochem Cytochem* 1962 *10* 348—354

FARRELL, K E, Fine structure of nerve fibres in smooth muscle of the vas deferens in normal and reserpinized rats *Nature* (Lond) 1968 *217* 279—281

FUXE, K., T HOKFELT and O NILSSON, A fluorescence and electron miscroscopic study on

face of the trough through staining solutions to the supporting film of a grid *J Ultrastruct Res* 1966 *14* 405—410

1966 *18* 387—399

iomic nervous system

York 2 U-1

of monoamines and

on of adrenergic and

Pharmacology of cholinergic and adrenergic transmission 1963 Eds

Oxford *3* 41—53

une neurons *Mechanisms on*

ell and B Uvnas 31—37

a granular fraction from the suprarenal medulla, containing the sympathomimetic catecholamines *Acta physiol scand* 1953 *29* 251—263

HOKFELT, T, The effect of reserpine on the intraneuronal vesicles of the rat vas deferens *Experientia* (Basel) 1966 *22* 56—57

HOKFELT, T, Ultrastructural studies on adrenergic nerve terminals in the albino rat iris after pharmacological and experimental treatment *Acta physiol scand* 1967a *69* 125—126

HOKFELT, T, On the ultrastructural localization of noradrenaline in the central nervous system of the rat *Z Zellforsch* 1967b *79* 110—117

HÖKFELT, T, *In vitro* studies on central and peripheral monoamine neurons at the ultrastructural level *Z Zellforsch* 1968 *91* 1—74

HOKFELT, T and G JONSSON, Studies on reaction and binding of monoamines after fixation and processing for electron microscopy with special reference to fixation with potassium permanganate *Histochemie* 1968 *16* 45—67

JARROT B and L L IVERSEN Subcellular distribution of monoamine oxidase activity in rat liver and vas deferens *Biochem Pharmacol* 1968 *17* 1619—1625

KAPELLER K and D MAYOR The accumulation of noradrenaline in constricted sympathetic nerves as studied by fluorescence and electron microscopy *Proc roy Soc* 1967 *167* 282—292

LUFT, H J, Improvements in epoxy resin embedding methods *J biophys biochem Cytol* 1961 *9* 409—414

MACHADO, A B M, Straight OsO_4 *versus* glutaraldehyde OsO_4 in sequence as fixatives for the granular vesicles in sympathetic axons of the rat pineal body *Stain Technol* 1967 *42* 293—300

MALMFORS, T Studies on adrenergic nerves The use of rat and mouse iris for direct observations on their physiology and pharmacology at cellular and subcellular levels *Acta*

NILS 1 The

NOR

revealed by histochemical studies on the intraneuronal distribution of the transmitter *Acta physiol scand* 1964 *63* Suppl 238 1—42

NORBERG K-A, M RITZEN and U UNGERSTEDT Histochemical studies on a special catecholamine-containing cell type in sympathetic ganglia *Acta physiol scand* 1966 *67* 260—270

PELLEGRINO DE IRALDI A and E DE ROBERTIS The neurotubular system of the axon and the origin of granulated and non granulated vesicles in regenerating nerves *Z Zellforsch* 1968 *87* 330—344

RENYI C and U S VON EULER The presence of histamine and noradrenaline in nerves as related to their content of myelinated and unmyelinated fibres *Acta psychiat scand* 1951 *26* 61—65

REYNOLDS, E S The use of lead citrate at high pH as an electron-opaque stain in microscopy *J Cell Biol* 1963 *17* 208—212

RICHARDSON, K C, The fine structure of the albino rabbit iris with special reference to th identification of adrenergic and cholinergic nerves and nerve endings in its intrinsic muscle *Amer J Anat* 1964 *114* 173—206
RICHARDSON, K C, Electron microscopic identification of autonomic nerve endings *Natur* (Lond) 1966 *210* 756
ROTH, R H, L STJÄRNE, F E BLOOM and N J GIARMAN, Light and heavy norepinephrin storage particles in the rat heart and in bovine splenic nerve *J Pharmacol exp Ther* 1968 *162* 203—212
SIEGRIST, G, F DE RIBAUPIERRE, M DOLIVO and CH ROUILLER, Les cellules chromaffine des ganglion cervicaux superieurs du rat *J Microscopie* 1966 *5* 791—794
STJÄRNE, L, Studies of noradrenaline biosynthesis in nerve tissue *Acta physiol scand* 1966 *67* 441—454
TAXI, J, Contribution a l'etude des connexions des neurons moteurs du système nerveu autonome *Ann Sci Nat* 1965 *7* 413—674
TRANZER, J P and H THOENEN, Various types of amine storing vesicles in periphera adrenergic nerve terminals *Experientia* (Basel) 1968 *209* 484—486
WOOD J G Electron microscopic localization of amines in central nervous tissue *Natur* (Lond) 1966 *209* 1131—1133

Acta physiol. scand 1969. 76. 441—445

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Energy Exchange in Isometric Contraction of Vascular Smooth Muscle Induced by K-ions

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Received 23 October 1968

Abstract

BEVIZ, A , L LUNDHOLM and E MOHME-LUNDHOLM *Energy exchange in isometric contraction of vascular muscle induced by K ions* Acta physiol scand 1969 *76* 441—445

[illegible] was relatively large in mesenteric arteries Therefore even on isometric contraction the muscle performed work on stretching of this component After correction of this internal work the ratio $\frac{L \cdot P}{H}$ was calculated to be 4 8, i e about 1/2 of that demonstrated by Hill (1958) in thermoelectrical determinations in isometrically contracted skeletal muscle from the frog In glucose free solution a significant correlation was found between the tension developed and the content of energy rich phosphate compounds in muscle

In a previous study (Beviz *et al* 1965) we found that on isometric contraction of bovine mesenteric arteries with adrenaline under anaerobic conditions, hydrolysis took place of energy rich phosphate compounds adenosine triphosphate = ATP, creatine phosphate = CrP) of an order of magnitude which agreed with that predicated by Hill (1949) from thermoelectric measurements on contracted muscle These determinations did not however, permit a more exact determination of the relation between tension development and energy consumption since the lactate production was not ascertained simultaneously

In further experiments it was found that adrenaline also stimulated the energy exchange in vascular muscle via a mechanism that was not connected with the contraction process (Mohme Lundholm and Vamos 1967) Finally it was found that vascular muscle had a large series elastic component which on maximal tension development (P_{max}) amounted to 15—20 % of the length of the vessel (L_0) (Lund

TABLE I Influence of K^+ on tension of and content of energy rich phosphate compounds in
Mean ± S E M Significance levels * = $P < 0.05$, ** = $P < 0.02$, *** = $P < 0.001$

Experimental conditions	ATP		CrP	
	Basal	Change	Basal	Change
145 meq/l K/ 0 glucose 1 min (n = 6)	0 33±0 11	—0 21±0 06**	0 50±0 13	—0 30±0 08*
145 meq/l K/ 0 5 % glucose 1 min (n = 5)	0 46±0 18	—0 29±0 12*	0 44±0 17	—0 18±0 10
145 meq/l K/ 0 glucose 3 min (n = 5)	0 25±0 08	—0 14±0 02***	0 39±0 04	—0 22±0 03**
145 meq/l K/ 5 % glucose min (n = 5)	0 56±0 07	—0 18±0 05*	0 56±0 08	—0 26±0 08*

holm and Mohme-Lundholm 1966) This meant that on maximal isometric" contraction the contractile elements became shortened by 15—20 % when they stretched the series-elastic component At the same time the muscle performed "internal work" which could be estimated approximately to be $\frac{P_{max}}{2} \cdot 0.2\ L_0$ In skeletal muscle the series elastic component was smaller — about 3—4 % of L_0 at P_{max} (Hill 1953) — and consequently its "internal work" on isometric contraction was less The energy exchange induced by the "internal work" can be assumed to be added to the energy exchange of the tension development The former component is 4—5 times larger in vascular muscle than in skeletal muscle and can therefore be expected to influence the relationship between tension development and energy consumption

In the study described below an attempt was made to take these factors into account in determination of the energy exchange on isometric contraction of vascular muscle A contraction was induced by K-ions which, to judge by previous experiments, do not, or only to a minor extent, directly stimulate the metabolism (Mohme-Lundholm and Vamos 1967) Experiments were also performed on preparations which had been suspended for 60 min in glucose-free solution in N_2 in an attempt to reduce the rate of resynthesizing processes of energy-rich phosphate compounds and also to decrease the basal lactate content of the preparations so that changes in this content could be more easily demonstrated A comparative study was also carried out on preparations suspended in 0 5 % glucose

bovine mesenteric artery Initial tension 18—20 p Concentration in μmoles/g wet weight

ATP + CrP		Lactate		Tension, increase
Basal	Change	Basal	Change	p
0.83±0.18	−0.51±0.15**	1.26±0.13	0.25±0.10*	80±17**
0.90±0.34	−0.46±0.19*	4.11±0.70	−0.72±0.66	97±10**
0.64±0.11	−0.34±0.06**	—	—	83±15**
1.13±0.10	−0.38±0.06**	—	—	85±15**

Method

The experiments were performed on bovine mesenteric arteries, with a diameter of 3.5—4.0 mm The ... after which 4 specimens were cut with a

between the points of attachment was 1.2 cm After 60 min the Tyrode solution was changed on two of the preparations to a solution where all Na ions in the Tyrode's solution were replaced by K (145 meq/l) The solutions, as before, were previously saturated with N_2 for 60 min and warmed to 37 C in one case the solution was glucose-free and in the other case contained 0.5 % glucose The specimens to which K Tyrode's solution had been added contracted rapidly and had generally attained a constant tension level after 1 min In one series they were frozen in freon 11 at −80° C 1 min after the addition of K+ Tyrode's solution and in another series 3 min after this addition The preparations were extracted with 6 % ice-cold perchloric acid and then analysed for their contents of ATP, CrP and lactate by methods described previously (Beviz *et al* 1965)

Results

The results are given in Table I In the experiments with glucose-free Tyrode's solution, 1 min after addition of K-ions the vascular muscle had, on the average, increased its tension from a mean initial value of 18 p to 98 p At the same time the ATP content decreased significantly by 0.21 μmoles/g wet weight and the CrP content by 0.30 μmoles/g, i.e there was a total decrease of the content energy-rich phosphate c... of 0.51 μmoles/g. The lactate content

in 5 out of 6 expts and, on the average, by 0.25 μmoles/g. In the presence of 0.5 % glucose essentially similar results were obtained. The total decrease of the content of energy-rich phosphate compounds amounted to 0.46 μmoles/g. The changes in the lactate content showed greater variation in this series and allowed no definite conclusions.

In order to obtain an idea of how rapidly the energy-rich phosphate compounds was resynthesized when the maximal tension development had been attained, a determination of the content of energy-rich phosphate compounds was also made 3 min after the addition of K^+. In these cases the vessel had a "restitution period" of about 2 min at a constantly raised tension. In previous tests we had found that the energy consumption was much smaller when the tension had reached a constant value than when it was raising (Lundholm and Mohme-Lundholm 1965). It is evident from Table 1 that the total decrease of ATP + CrP was 0.34 μmoles/g after 3 min in the absence of glucose, that is 0.17 μmoles/g less than after 1 min. The increase in tension was then of almost exactly the same magnitude as 1 min after the addition of K^+. Some resynthesis of energy-rich phosphate compounds can be assumed to have occurred during the interval (2 min) but this was probably more rapid than about 0.08 μmoles/min.

Discussion

In a previous study the energy consumed on the production of tension was calculated from Hill's (1949) formula $\frac{L \cdot P}{H}$, where L was the length of the muscle in cm, P the development of tension in p and H the energy consumption in pcm (Beviz et al. 1965). In the experiments with a contraction time of 1 min in glucose-free solution the total hydrolysis of ATP and CrP was 0.51 μmoles/g. In addition there was a lactate increase of 0.25 μmoles/g. In the production of 1 mol lactate from glycogen or hexose monophosphate esters, 1.5 mol of energy rich phosphate esters are formed. The total consumption of energy-rich phosphate esters can therefore be calculated to be 0.89 μmoles/g. On hydrolysis of 1 mol ATP, 8400 cal are liberated, and since the mechanical heat equivalent is $4.26 \cdot 10^4$, the hydrolysis of the energy-rich phosphate compounds corresponds to an energy exchange of 318 pcm/g muscle. However, the muscle performed work on stretching of the series-elastic component. Experiments with "quick release" (Lundholm and Mohme-Lundholm 1966) showed that this stretching at the developed tension amounted to about 10 % of the length of the muscle. The tension development was calculated to be 1066 p/cm² when the cross-section surface area was about 0.075 cm². The work per cm muscle per cm² required for stretching of the series-elastic component can be calculated approximately to be $\frac{1066}{2} \cdot 0.1 \cdot 1 = 53$ pcm. If the energy consumption is corrected with regard to this work the ratio $\frac{L \cdot P}{H}$ is calculated to be $\frac{1066 \cdot 1.2}{318-53} = 4.8$. This value

is approximately half that found by Hill (1958) in thermoelectrical measurements on isometrically contracted skeletal muscle from the frog It is in good agreement with the preliminary calculation of the corresponding value which we made previosly when the effect of lactate formation was included (Beviz *et al* 1965)

Since the development of tension in vascular muscle is accompanied by hydrolysis of energy rich phosphate compounds, it seemed reasonable to assume that the tension development should be dependent upon the available ATP and CrP in the muscle A significant correlation was also found in the experiments with glucose free Tyrode s solution, between the magnitude of the final tension and the total content of energy rich phosphate compounds ATP + CrP at the end of the contraction The coefficient of correlation was 0 57 (n = 10 P < 0 05) This may indicate that the final tension attained by a substrate depleted muscle is limited by the decreasing content of energy rich phosphate compounds On the other hand no corresponding correlation was found in glucose containing medium The content of energy rich phosphate compounds was however somewhat higher, on the average in glucose containing than in glucose free solution (Table I)

References

BEVIZ A L LUNDHOLM E MOHME LUNDHOLM and N VAMOS Hydrolysis of adenosinetriphosphate and creatinephosphate on isometric contraction of vascular smooth muscle *Acta physiol scand* 1965 *65* 268—272

HILL, A V [illegible]

HILL A [illegible]

HILL A [illegible]
Proc roy [illegible]

LUNDHOLM L and E MOHME LUNDHOLM Energetics of isometric and isotonic contraction in isolated vascular smooth muscle under anaerobic conditions *Acta physiol scand* 1965 *64* 275—282

LUNDHOLM L and E MOHME LUNDHOLM Length at inactivated contractile elements length tension diagram active state and tone of vascular smooth muscle *Acta physiol scand* 1966 *68* 347—359

MOHME LUNDHOLM E and N VAMOS Influence of Ca deficiency and pronethalol on metabolic and contractile effects of catecholamines and K ions in vascular muscle *Acta pharmacol (Kbh* 19 7 *25* 87—96

Acta physiol. scand 1969. 76 446—457
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Release of Histamine and Formation of Smooth-Muscle Stimulating Principles in Guinea-Pig Lung Tissue Induced by Antigen and Bee Venom Phosphatidase A

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Received 24 October 1968

Abstract

Fredholm, B and K. Strandberg *Release of histamine and formation of smooth muscle stimulating principles in guinea pig lung tissue induced by antigen and bee venom phosphatidase a* Acta physiol scand 1969 76 446—457

of histamine and formation of smooth muscle stimulating principles (spasmogenic lipids) occurred when minced guinea pig lung tissue was exposed to antigen or to bee venom phosphatidase A In agreement with earlier reports the anaphylactic histamine release was a rapid process, and it could be inhibited by anoxia in the absence of glucose and by potassium cyanide ninhydrin and N-ethyl maleimide The histamine release induced by phosphatidase A was not influenced by these inhibitors or by anoxia and the release was slower than in anaphylaxis The spasmogenic lipids formed had similar chromatographic and biological properties to those reported for the corresponding principles formed in anaphylaxis and the process of formation was inhibited by oxygen lack and by the above enzyme inhibitors One noteworthy feature of the spasmogenic lipid formation was that the inhibition caused by anoxia could not be counteracted by glucose The results support earlier hypotheses regarding the mode of action of phosphatidase A on guinea pig lung tissue It is suggested that mast cells are not [illegible]

The histamine releasing capacity of bee venom first observed by Feldberg and Kellaway (1937) can be attributed to at least three principles These are phosphatidase A, which is believed to act via the formation of lysophosphatides in the tissue (Feldberg and Kellaway 1938 Rothschild 1965) melittin probably acting by virtue of its high surface activity (Riley 1958, Rothschild 1965) and a recently described polypeptide fraction *F II which is thought to initiate an energy requiring* histamine release process in rat mast cells resembling that induced by compound 48/80 (Fredholm and Haegermark 1967)

A preliminary report of this investigation was presented at the XXI Scandinavian Pharmacological Meeting Copenhagen, Denmark 1967

Lipid soluble, smooth muscle stimulating principles have been shown to appear in various tissues exposed to phosphatidase A containing venoms (for references see Vogt 1965), antigen or other histamine releasing agents (Chakravarty, Högberg and Uvnas 1959, Chakravarty 1960 b, Anggård *et al* 1963) Such principles have often been referred to as "slow reacting substance, SRS Studies on the smooth muscle stimulating principles from guinea pig lung (antigen), rat mast cells (compound 48/80), and cat paw (compound 48/80) have revealed that they consist of mixtures of compounds with different chemical and biological properties (Anggård *et al* 1963) In the present work the term spasmogenic lipids' will be used to denote acid lipid soluble smooth muscle stimulating principles which have not been further characterized chemically or biologically

Although both the release of histamine and the formation of spasmogenic lipids induced by bee venom in guinea pig lung have been ascribed to phosphatidase A, the effects of purified enzyme have only been sparsely investigated In a few studies has the appearance of both histamine and spasmogenic lipids been determined (Middleton and Phillips 1964), and the results do not allow any conclusions to be drawn about the possible correlation of the mechanisms involved This is in contrast to anaphylaxis, where the corresponding mechanisms have been systematically studied and shown to have several features in common (Boréus and Chakravarty 1960, Chakravarty and Uvnas 1960)

The present work was designed as a comparative study of the anaphylactic and phosphatidase A induced release of histamine and the formation of spasmogenic lipids, and of the relationship between the two processes induced by either releaser

Methods

Animals

Male guinea pigs (200—300 g) were given, on the same day one subcutaneous and one intraperitoneal injection each of 100 mg egg albumin dissolved in 1 0 ml of saline Three days later the intraperitoneal injection was repeated The animals were used 4—16 weeks later for anaphylactic reaction in minced lung tissue

Incubation procedure

The technique was adopted from Ungar and Parrot (1936), as modified by Chakravarty [illegible]

A 0 5-ml sample of the supernatant was removed from each tube for histamine assay The rest of the supernatant in the filtrate tubes was used for the assay of spasmogenic lipids

[illegible] release process nitrogen or oxygen was [illegible] period of 15 min After addition of the [illegible] sed above the level of the liquid The [illegible] by the oxygen or nitrogen treatment [illegible] ed with the tissue for 15 min before

Assay of histamine and spasmogenic lipids

Histamine was determined fluorometrically according to Shore, Burkhalter and Cohn (1959), using an Aminco Bowman spectrophotofluorometer and 10 mm cuvettes It was measured in both filtrate and tissue extracts, and the release was expressed as a percentage of the total content of histamine in each sample This amount was usually 7—15 μg/g lung tissue (wet weight) The spontaneous release after 20 min incubation at 37° C was 3 3±0 39 (M±SEM n=18)

Spasmo ... pH 3 with ... ex-tracted ... d to pH 8 I ... the addition of 1 N NaOH After extraction, the extracts were evaporated free from ether and neutralized by the addition of 1 N HCl

The recovery of active material in this extraction procedure is about 70 per cent, whether determined for spasmogenic lipids produced by compound 48/80 in cat paw (Strandberg and Uvnas, to be published) or by antigen in guinea pig lung tissue (Chakravarty 1960 b, Strandberg, unpublished)

The spasmogenic activity of the extracted (lipid soluble) material was routinely determined on guinea pig ileum in the presence of atropine sulphate (1 μg/ml) and mepyramine maleate (1 μg/ml) Generally a four point assay technique was used (Chakravarty 1959), but in some instances, due to low activity, close-bracket assays had to be performed The values presented are expressed as spasmogenic lipids, units per gram lung tissue, referring to a standard of cat paw SRS (Strandberg to be published), and are not corrected for the extraction losses

Characterization of spasmogenic lipids

The criteria used for characterization of the spasmogenic lipids were the behaviour on silicic acid, column and thin layer (TLC), chromatography, and effects in different biological assays (guinea-pig ileum, rat uterus and rabbit duodenum) The column chromatography was per...d according to Änggård *et al* (1963), details of the other techniques used are described elsewhere (Strandberg, to be published)

Materials

A *bee venom preparation* (referred to as "bee venom") obtained by precipitation with picric ... usly supplied by Dr B Hogberg ... *I II* were obtained by gel filtra- ... ously (Fredholm and Haegermark ... II contained a basic polypeptide

Prostaglandin $F_{1\alpha}$ was a gift from Prof S Bergstrom Dept of Chemistry Karolinska Institutet, Stockholm Sweden

Results

Dose-response relationships

It was established that antigen did not release histamine or induce the formation of spasmogenic lipids in lung tissue from non-sensitized guinea pigs and that the venom and venom fractions elicited the same response in sensitized as in non-sensitized lung tissue

In one series of experiments the histamine release was determined after 20 min incubation of the lung tissue with the releasing agent (Fig 1 a) Bee venom released histamine already at a concentration of 2 μg/ml and with 100 μg/ml 25—30 per cent of the lung histamine was liberated The effect of the phospholipidase A-containing fraction (F I) was similar to that of unfractionated venom The fraction F II, capable of releasing histamine from rat tissues was included in the experiments, and was found to have a negligible effect even at the highest concentration used As the studies of the time course revealed that the release process induced by

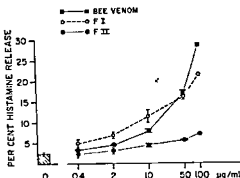


Fig 1 a Concentration — response curves for histamine release from lung tissue induced by bee venom, F I and F II at 37° C Incubation time 20 min Each point represents the mean of 3 expts, except for the 100 μg values, which are based on 2 expts Shaded column denotes spontaneous release, vertical bars standard errors

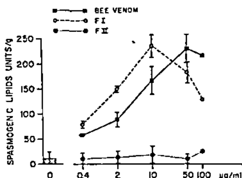


Fig 1 b Concentration — response curves for formation of spasmogenic lipids from lung tissue induced by bee venom, F I and F II at 37° C Incubation time 20 min Each point represents the mean of 3 expts, except for the 100 μg values, which are based on 2 expts Vertical bars indicate standard errors Shaded column denotes spontaneous formation, vertical bars standard errors

F I was very slow, the experiments were repeated with prolonged incubation periods After 160 min incubation the release induced by F I had increased, *e g* 100 μg/ml had liberated 40—50 per cent of the histamine, while the effect of F II was still no greater than after 20 min

Spasmogenic lipids were formed by the phosphatidase A-containing fraction (F I) and by bee venom but were not detected in samples exposed to F II (Fig 1 b) In these experiments the incubation period was 20 min The optimal concentration for production of spasmogenic lipids by bee venom was 50 μg/ml, and by F I 10 μg/ml

Time course of the reactions

The release processes were followed for 160 min after the addition of releaser In confirmation of earlier results (Brocklehurst 1960 Chakravarty 1960 b) the antigen-induced release of histamine was found to occur rapidly After 5 min which was the shortest period studied egg albumin, 1 mg/ml had liberated 25—30 per cent of the histamine and no further release took place during prolonged incubation With a lower dose 0 1 mg/ml less histamine was released but the process was as fast as with the higher concentration (Fig 2 a) The time course of the histamine release induced by the phosphatidase A-containing fraction (F I) had a different appearance The release proceeded slowly almost linearly, throughout the incubation period and after 160 min about 40 per cent of the histamine had been released by F I 25 μg/ml (Fig 2 a)

The appearance of spasmogenic lipids in the incubation fluid—whether induced by antigen or F I—occurred within the first 10—20 min of incubation After this time there was a continuous and marked decrease in the amount of spasmogenic lipids found in the supernatant fluid (Fig 2 b)

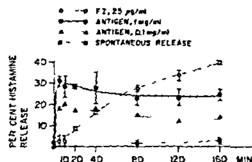


Fig 2 a. Time course of histamine release from lung tissue induced by antigen, 0 1 and 1 0 mg/ml, and F I, 25 µg/ml Means and ranges of 2 expts., except for antigen, 0 1 mg/ml, which is 1 expt

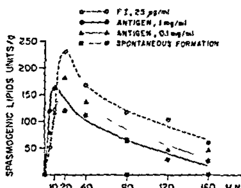


Fig 2 b Time course of the formation of spasmogenic lipids from lung tissue induced by antigen, 0 1 and 1 0 mg/ml, and F I, 25 µg/ml

Influence of temperature

It has been shown that antigen does not induce release of histamine or formation of spasmogenic lipids in guinea-pig lung tissue heated at 43° C or above (Mongar and Schild 1957 b Chakravarty and Uvnas 1960) In one series of experiments the lung tissue was incubated with antigen or the phosphatidase A-containing fraction (F I) for 20 min at different temperatures At 0° C only a slight histamine release was obtained At 22° C and still more at 37° C, the effect of both agents was increased At 45 C antigen released practically no histamine while F I was more active than at 37 C (Fig 3 a) The formation of spasmogenic lipids showed the same temperature dependence as the release of histamine with the exception that with F I less spasmogenic lipids were found after incubation at 45° C than at 37° C (Fig 3 b)

In another series of experiments the lung tissue was preincubated in buffered salt solution at 37° C or 45° C for 20 min prior to the equilibration (10 min) and incubation (20 min) periods at 37° C It was found that preincubation at 45° C caused a pronounced decrease in the subsequent release of both histamine and spasmogenic lipids caused by antigen while the same treatment had no effect on the F I-induced release (Fig 4)

Influence of anoxia and glucose

Lung tissue was incubated with liberator for 40 min under oxygen or nitrogen in the presence or absence of glucose 5 6 mM (Fig 5 a b) Antigen, 1 mg/ml and F I, 25 µg/ml, both liberated 14 per cent of the lung histamine in aerobic milieu in the absence of glucose Other figures were calculated as percentages of this value In confirmation of earlier results (Diamant 1962) the antigen induced histamine release was almost completely blocked by anoxia in a glucose-free medium but in the presence of glucose the inhibition was only partial In aerobic milieu the presence

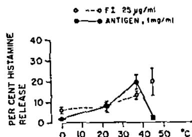


Fig 3 a Influence of temperature on the release of histamine from lung tissue induced by antigen and F I Means and ranges of 2 expts Spontaneous release deducted

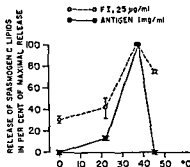


Fig 3 b Influence of temperature on the formation of spasmogenic lipids in lung tissue induced by antigen and F I The values are expressed as percentages of the formation at 37° C, in the figure denoted by "maximal release" Means and ranges of 2 expts Spontaneous release deducted

of glucose increased the release above the value in glucose-free buffer The F I-induced release was not significantly influenced by such changes in the milieu (Fig 5 a)

The effects of anoxia and glucose on the formation of spasmogenic lipids were at variance with the influence of those factors on the release of histamine Firstly, not only the anaphylactic but also the F I-induced formation was depressed in the absence of oxygen, although the latter to a lesser degree Secondly, the blockade of neither the anaphylactic nor the F I-induced formation could be counteracted by glucose, 5 6 mM (Fig 5 b)

Effects of enzyme inhibitors

Potassium cyanide, *N*-ethylmaleimide and ninhydrin were selected for the study All these agents caused an inhibition of the anaphylactic histamine release which in the case of potassium cyanide and *N*-ethylmaleimide is in agreement with previous

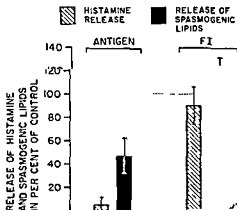


Fig 4 Release of histamine and formation of spasmogenic lipids from lung tissue preincubated at 45° C Concentration of antigen 1 mg/ml F I 50 µg/ml The results are expressed as percentages of the release from tissue preincubated at 37° C Means and standard errors of 4) expts The spontaneous release was deducted before calculation

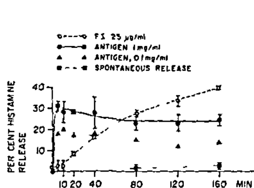


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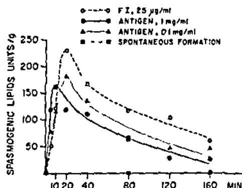


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This shape of a time-curve may be explained by a slow penetration of the releaser to the site of action and/or by a slow progress of the reactions induced by it. To what extent delays in penetration of the phosphatidase A molecule contribute to the slow release cannot be stated. However, in perfusion experiments, where the delay due to penetration can be expected to be reduced, the release induced by bee venom continues for more than three hours (Feldberg and Kellaway 1937). This observation seems to reduce the importance of the penetration and thus lend support to the idea that the rate limiting step is the release process *per se*. Whether this process—as suggested—consists of lysis of mast cells caused by lysophosphatides, remains to be elucidated. The idea seems justified, because similar time-curves can be obtained for the action of lysolecithin (in low concentration) on brain homogenates (Webster 1957).

Several authors have observed a correlation between the histamine release and the formation of spasmogenic lipids in anaphylaxis, some reports also include a correlation between the amounts of substances released and the number of mast cells in the tissue (Boreus and Chakravarty 1960, Eilbeck and Smith 1967). This has led to the suggestion that the release of both histamine and spasmogenic lipids is the result of a common mechanism, and that the mast cell is the source of both. Part of the evidence is similarities observed in the energy-dependence of the release processes. The present results have to some extent confirmed the earlier finding that the anaphylactic release of histamine and spasmogenic lipids are equally dependent on metabolic energy. One additional observation was made however, while the inhibition of the anaphylactic histamine release brought about by anoxia could partly be counteracted by glucose, which is in agreement with earlier observations (Diamant 1962), this was not the case with the formation of spasmogenic lipids. It should be noted in this connection that the formation of spasmogenic lipids induced by the phosphatidase A containing fraction was also markedly depressed by anoxia both in the presence and absence of glucose in spite of the fact that the release of histamine by F I was not affected by oxygen lack. These facts suggest that there is some step in the production of spasmogenic lipids induced by both antigen and F I which is dependent on oxygen.

Earlier information about the possible correlation between venom induced release of histamine and formation of spasmogenic lipids is very scanty. In some tissues exposed to *Naja naja* venom, a high histamine release was accompanied by the formation of substantial quantities of spasmogenic lipids while in others it was not (Middleton and Phillips 1964). In the present study no evident correlation between the two processes induced by phosphatidase A was found. Not only was the rate of the formation of spasmogenic lipids much faster than the release of histamine but differences were also revealed in the influence of temperature and enzyme inhibitors and—as mentioned above in the oxygen dependence. When interpreting the results it should be remembered that whereas histamine seems to be very stable under the prevailing conditions the spasmogenic lipids are subjected to inactivation as pointed out by Chakravarty (1960 b). It is the amount assayed

amount formed less that inactivated An obvious demonstration of this inactivation is the pronounced decrease of spasmogenic lipids found after prolonged incubation (*vide* time curves) However, taking into consideration possible errors in the interpretation thus introduced the results still seem to indicate definite dissimilarities between the courses of the two processes

The observation that the formation of spasmogenic lipids occurs much more rapidly than the release of histamine makes it even questionable whether mast cells are necessarily involved in the formation of spasmogenic lipids induced by phosphatidase A We do not consider this statement contradictory to the earlier observation that spasmogenic lipids—with similar characteristics to guinea pig spasmogenic lipids—can be formed from an almost pure suspension of rat mast cells (Änggård *et al* 1963) Instead, it seems reasonable to assume that spasmogenic lipid precursors are localized to various elements of the tissue and, regardless of origin can be transformed to biologically active compounds under the influence of histamine releasing agents As in lung tissue the mast cells represent only a minor part of the tissue their contribution to the total output of spasmogenic lipids can be expected to be small as compared to the non mast cell spasmogenic lipids

Spasmogenic lipids formed in guinea pig lung tissue on exposure to bee venom or antigen have been separated into compounds with different biological properties y silicic acid chromatography (Strandberg to be published) In that work the lution profiles were generally qualitatively similar regardless of releasing agent although quantitative differences in the distribution of the spasmogenic activity were encountered The present study has shown that the action of purified phosphatidase A also leads to the formation of compounds with similar properties This lends weight to the earlier suggestion (see Vogt 1965) that spasmogenic lipids may derive from tissue phosphatides

Note added in proof Recently Breithaupt and Habermann (1968) presented important new information about the polypeptide contained in F II

This investigation was supported by grants from Karolinska Institutet from Svenska Sällskapet for Medicinsk Forskning (to K S) and from the National Institute of Health U S A (Grant No. AM 04063 08)

The skilful technical assistance of Mrs Kerstin Annell Mrs Birgitta Ask and Miss Ann Christine Jarntos is gratefully acknowledged

References

Änggård E L Bergqvist B Högberg K Johansson I L Thon and B Uvnäs Bo- ... release from cat paw guinea pig lung and ... 97 110 ... in chopped guinea pig lung I Effect ... 1961 *113* 521 539 ... the rat hamster and guinea pig *Acta*

Boréus L O and N Chakravarty Tissue mast cells histamine and slow reacting substance in anaphylactic reaction in guinea pig *Acta physiol scand* 1960 *48* 315—322

Breithaupt H and E Habermann Mastzelldegranulierendes Peptid (MCD Peptid) aus Bienengift Isolierung biochemische und pharmakologische Eigenschaften *Arch Pharmak exp Path* 1968 *261* 252—270

Brocklehurst W E The release of histamine and formation of a slow reacting substance (SRS-A) during anaphylactic shock *J Physiol* (Lond 1960 *151* 416—435

Chakravarty N A method for the assay of slow reacting substance *Acta physiol scand* 1959 *46* 298—313

CHAKRAVARTY, N, The mechanism of histamine release in anaphylactic reaction in guinea pig and rat *Acta physiol scand* 1960a *48* 133—145

CHAKRAVARTY, N, The occurrence of a lipid soluble smooth-muscle stimulating principle ("SRS") in anaphylactic reaction *Acta physiol scand* 1960b *48* 167—177

CHAKRAVARTY, N, B HOGBERG and B UVNAS, Mechanism of the release by compound 48/80 of histamine and of a lipid soluble smooth muscle stimulating principle, ("SRS"). *Acta physiol scand* 1959 *45* 255—270

CHAKRAVARTY, N and B UVNÄS, Histamine and a lipid-soluble smooth muscle stimulating principle ("SRS") in anaphylactic reaction *Acta physiol scand* 1960 *48* 302—314

DIAMANT, B, Observations on anaphylactic and compound 48/80-induced histamine release from guinea pig and rat lung tissue *in vitro* *Acta physiol scand* 1962 *55* 11—25

EILBECK, J F and W G SMITH, Histamine release from the mast cells of guinea pig lung *J Pharm Pharmacol* 1967 *19* 374—382

[illegible] ine liberator compound 48/80 in [illegible]

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FELDBERG, W and C H KELLAWAY, Liberation of histamine and formation of lysocithin like substances by cobra venom *J Physiol* (Lond) 1938 *94* 187—226

FELDBERG, W, H F HOLDEN and C H KELLAWAY, The formation of lysocithin and of a muscle stimulating substance by snake venoms *J Physiol* (Lond) 1938 *94* 232—248

FINKE, S R, G B PHILLIPS and E MIDDLETON, JR, Histamine release from human leukocytes by lysolecithin and the effect of *in vitro* anaphylaxis on leukocyte lipids *J Lab clin Med* 1966 *67* 601—609

[illegible] *armacol*

[illegible] a mast

[illegible] venom fractions and compound 48/80 on skin and lung tissue of the rat *Acta physiol scand* 1969 *76* 288—298

HÖGBERG, B and B UVNÄS, The mechanism of the disruption of mast cells produced by compound 48/80 *Acta physiol scand* 1957 *41* 345—369

MAY, B, C HOLLER and E WESTERMANN, Über die Bedeutung der Phospholipase A fur die histaminfreisetzende Wirkung des Cobragiftes *Arch Pharmak exp Path* 1967 *256* 237—256

MIDDLETON, E, JR, and G B PHILLIPS Distribution and properties of anaphylactic and venom induced slow reacting substance and histamine in guinea pigs *J Immunol* 1964 *93* 220—227

MONGAR, J L and H O SCHILD, Inhibition of the anaphylactic reaction *J Physiol* (Lond) 1957a *135* 301—319

MONGAR, J L and H O SCHILD, Effect of temperature on the anaphylactic reaction *J Physiol* (Lond) 1957b *135* 320—338

MOTA I and I VUGMAN Action of compound 48/80 on the mast cells and histamine content of guinea pig tissues *Brit J Pharmacol* 1956 *11* 304—307

MOUSSATCHE H and A PROUVOST DANON Influence of oxidative phosphorylation inhibitors on the histamine release in the anaphylactic reaction *in vitro* *Experientia* (Basel) 1958 *14* 414—415

PARROT, J L Sur la reaction cellulaire de l anaphylaxie Son caractere aerobie *C R Soc Biol* (Paris) 1942 *136* 361—362

RILEY, J F The mechanism of histamine release from mast cells *J Pharm Pharmacol* 1958 *10* 272

ROTHSCHILD A M Histamine release by bee venom phospholipase A and mellitin in the rat *Brit J Pharmacol* 1965 *25* 59—66

SHORE, P A A BURKHALTER and V H COHN JR A method for the fluorometric assay of histamine in tissues *J Pharmacol* 1959 *127* 182—186

TRETHEWIE E R Comparison of hemolysis and liberation of histamine by two Australian snake venoms *Austr J exp Biol Med* 1939 *17* 145 155

UNGAR, G and J PARROT Recherches sur le choc anaphylactique *in vitro* Mise en liberte d une substance active par le poumon isole du cobaye sensibilise *C R Soc Biol* (Paris) 1936 *123* 676—678

WEBSTER, G R Clearing action of lysolecithin on brain homogenates *Nature* (Lond) 1957 *180* 660—661

VOGT, W Liberation of pharmacologically active lipids by enzymes contained in veno[illegible] In *Recent Advances in the* [illegible] pp 43—51

Acta physiol scand. 1969 76 458—462

From the Department of Food Hygiene National Institute of Public Health Stockholm the Department of Surgery, University Hospital and the Department of Physiology University of Uppsala Sweden

Renal Clearances of Labelled Inulin (Inulin-Carboxyl-^{14}C, Inulin-Methoxy-^{3}H) and a Polyethylene Glycol (PEG 1000) in the Rat

By

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Received 28 October 1968

Abstract

BERGLUND F A ENGBERG E PERSSON and H ULFENDAHL. *Renal clearances of labelled inulin (inulin-carboxyl ^{14}C, inulin methoxy ^{3}H) and a polyethylene glycol (PEG 1000) in the rat* Acta physiol scand 1969 76 458—462

The simultaneous renal clearances of ^{14}C-inulin ^{3}H inulin and PEG 1000 were measured in 10 unanesthetized male Sprague Dawley rats by a constant infusion technique Both the ^{3}H inulin/PEG 1000 and the ^{3}H inulin/^{14}C-inulin clearance ratios were significantly below unity There was a negative correlation between ^{3}H inulin/PEG 1000 or ^{14}C-inulin/PEG 1000 clearance ratios and the clearance of PEG 1000 indicating more efficient "sieving" at increased filtration rates Inulin (unlabelled as well as labelled derivatives) is considered unsuitable for the accurate measurement of glomerular filtration rate in the rat

Inulin clearance is generally accepted as a measure of glomerular filtration rate (GFR) but certain objections have been raised from time to time Morphological and physiological studies indicate retention of inulin in the kidneys (Falbriard 1967) possibly due to reabsorption of filtered inulin by the tubules Such reabsorption seems to be negligeable from a quantitative viewpoint (cf Thurau Valtin and Schnermann 1968) On the other hand the varying physico-chemical properties of inulin may influence the filterability through the glomerular membrane (Berglund 1965) as well as through other membranes (Chen Terepka and Lane 1963 Chen and Lane (1964) also showed a more rapid penetration of inulin ^{14}COOH than of non labelled inulin from plasma to thoracic duct lymph in the dog Thus inulin and its labelled derivatives may filter at different rates and their renal clearances may therefore deviate more or less from the true GFR In the rat the clearance of unlabelled inulin is lower than that of PEG 1000 (Berglund 1965) It seemed to be of interest therefore, to compare the simultaneous clearances of different labelled inulins with that of PEG 1000 in the rat The results showed significant differences between the clearances of ^{3}H inulin ^{14}C-inulin and PEG 1000

Methods

Male Sprague Dawley rats, weight 210—310 g were used They were given standard pellet food and water *ad libitum* At least 5 days prior to each experiment, catheters were inserted into the abdominal aorta superior caval vein and urinary bladder to allow clearance measurements with minimal restriction and without anesthesia (Engberg 1969) No food or water was available during the 3 hr experimental procedure Test substances were administered i v by constant infusion after a priming dose Each experiment comprised five 30 min clearance periods

The follo [illegible] *1000* (British Drug Houses L [illegible] and Nuclear Corp, Mass, L [illegible] ^{3}H ^{3}*H* inulin (New Eng la [illegible] ical source of the inulin is not known to us

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and scintillation liquid (0 7 % butyl PBD in toluene) was added The activities of ^{14}C and of ^{3}H were then measured in a Packard liquid scintillation counter with manually adjusted channels The results were calculated according to the two channel method (Danielson, Delahayes and Sjostrand 1966) *In vitro* tests after addition of known amounts of the labelled inulins showed identical measuring efficiency of inulin in serum and urine

Statistical analysis was done by standard methods as presented by Snedecor (1956)

Results

The clearance of PEG 1000 was $0\,97 \pm 0\,20$ mg/min/100 g b w Both the ^{3}H inulin/PEG 1000 and the ^{3}H inulin/^{14}C inulin clearance ratios were significantly below 1 0 (Table I) There was a negative correlation between the ^{3}H inulin/PEG 1000 or ^{14}C inulin/PEG 1000 clearance ratios and the clearance of PEG 1000 (Fig 1)

Discussion

The clearance of PEG 1000 in our experiments was of the same order of magnitude as reported earlier in blackhooded rats with mannitol diuresis ($1\,00 \pm 0\,25$ ml/min/100 g b w $n = 17$ Berglund 1964 $1\,03 \pm 0\,18$ ml/min/100 g b w $n = 19$ Berglund 1965) As the clearance of both PEG 1000 and PEG 1500 is very close to that of mannitol in the rat (Berglund 1968) PEG 1000 clearance seems to be a valid measure of GFR in this species

The low ^{3}H inulin/PEG 1000 clearance ratio ($0\,69 \pm 0\,11$) in our experiments [illegible] even lower than the inulin/PEG 1000 clearance ratio ($0\,81 \pm 0\,14$) reported by Berglund (1965) The low clearance ratio is most probably due to a low filtrability of large molecular weight fractions of the inulin preparations Molecular d[illegible] of inulin has been well documented by various physico-chemical method[illegible] 1965) Also the pore size of the glomerular membrane seems to be smaller [illegible] than in the dog This has been demonstrated in experiments with [illegible] molecular sizes in the two species (Berglund 1968 — In our experime[illegible] of ^{3}H inulin was significantly lower than that of ^{14}C inulin Th[illegible] higher filterability of the ^{14}C inulin than of the ^{3}H inulin It sh[illegible] ever that different lots of ^{14}C inulin m[illegible] show marked diffe[illegible]

TABLE I Renal clearances of ^{14}C-inulin, ^{3}H-inulin and PEG 1000 in 10 rat experiments

Exp	Weight g	Clearance			Clearance ratio		
		^{14}C ml/min/100 g	^{3}H	PEG	$^{14}C/^{3}H$	$^{14}C/PEG$	$^{3}H/PEG$
1	211	0 86	0 62	0 83	1 37	1 05	0 77
2	226	0 98	0 75	1 05	1 30	0 94	0 72
3	230	0 86	0 63	0 82	1 38	1 07	0 78
4	232	0 85	0 77	1 30	1 13	0 66	0 60
5	234	0 80	0 67	1 12	1 23	0 73	0 59
6	276	0 69	0 59	0 65	1 35	1 09	0 88
7	216	0 87	0 58	0 80	1 46	1 06	0 73
8	292	0 83	0 59	0 91	1 43	0 93	0 66
9	306	0 94	0 59	1 16	1 60	0 82	0 52
10	304	0 63	0 67	1 06	1 02	0 59	0 61

Clearances (mean ± standard deviation)

^{14}C-inulin 0 83 ± 0 105 ml min 100 g body wt
^{3}H inulin 0 65 ± 0 068 » »
1000 0 97 ± 0 200 » »

earances ratios (mean ± standard deviation)

^{3}H PEG 1000 0 69 ± 0 11 p 0 001
^{14}C PEG 1000 0 89 ± 0 18 p 0 10
^{3}H ^{14}C 0 79 ± 0 12 $p < 0\ 001$
(p calculated for obtained ratios versus 1 0)

Regression equations for clearance ratios against C_{PEG}

^{3}H PEG = 1 16—0 48 C_{PEG} r = — 0 88
^{14}C/PEG = 1 65—0 78 C_{PEG} r = 0 85
^{14}C/^{3}H = 1 65—0 33 C_{PEG} r = — 0 40

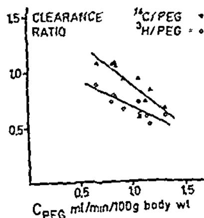


Fig 1 Relationship between clearance ratios and the clearance of PEG 1000 (C_{PEG}) in 10 rat experiments. Regression equations are given in Table I

through cellophane membranes (Chen and Lane 1964) Our experiments therefore do not prove any general advantage of ^{14}C inulin over ^{3}H inulin for the measurement of GFR in the rat.

Our data also showed a marked decline of inulin/PEG 1000 ratios at high clearances of PEG 1000 This relationship was not evident in individual rats and not in the earlier paper by Berglund (1965) From a theoretical viewpoint, however, one might expect a lower clearance ratio equivalent to increased 'sieving', at increased filtration rates (Renkin 1954), although not necessarily following a straight line, as calculated here over a narrow range of glomerular filtration rates

The low inulin/PEG 1000 clearance ratios, their dependence on GFR, and the poorly defined molecular weight of inulin, preclude the use of inulin or its labelled derivatives for accurate measurement of GFR in the rat The low clearance of inulin in the rat may cause serious errors in studies of renal function This is most conspicuous in the measurement of the tubular reabsorption (T) of any substance (X) at varying plasma levels (P_X) The reabsorption equals the difference between filtered load and excreted load *i e* $T = P_X \cdot GFR - U_X \cdot V$ The latter term is practically zero until the transfer maximum (Tm) of reabsorption is reached, but will then increase rapidly with increasing plasma levels The calculated term $P_X \cdot GFR$ will be erroneously low if GFR is measured with inulin and the error (*e g* as µmoles/min) will increase with increasing values of P_X The error of the calculated reabsorption (T) will therefore also increase at higher plasma levels of X, yielding a false pattern of reabsorption at low plasma levels and secretion at high plasma levels This was pointed out in an earlier paper on sulfate reabsorption in the rat (Berglund 1964) The same source of error is probably responsible for the reports of a reabsorption—secretion pattern for magnesium (Averill and Heaton 1966) and on a decreasing reabsorption of phosphate at high plasma levels (Frick 1968) in the rat

This project was supported by a grant from Pharmacia AB and grant B 67 14X 10 03 from the Swedish Medical Research Council The experimental work was largely done at the research division Pharmacia AB Uppsala and at the Department of Pharmacology Karolinska Institutet Stockholm We thank Mrs Stina Antonsson Mr Ove Forsberg Miss Christina Korner and Mrs Margareta Sandstrom for skillful technical assistance

References

AVERILL C M and F H HEATON The renal handling of magnesium *Clin Sci* 1966 *31* 353—360

BERGLUND F Reabsorption of inorganic sulfate by the renal tubules of the rat *Acta physiol scand* 1964 62 336—343

BERGLUND F Renal clearances of inulin polyfructosan S and a polyethylene glycol (PEG 1000) in the rat *Acta physiol scand* 1965 *64* 238—244

BERGLUND F Renal excretion and volume of distribution of polyethylene glycol (PEG in the rat and the dog *Acta physiol scand* 1968 *73* 1 2 0 A

CHEN P S JR and K LANE Specific activity of inulin $C^{14}OOH$ in serum and lymph of nephrectomized dog *Proc Soc exp Biol* N Y 1964 *116* 40—42

CHEN P S JR A R TEREPKA and K LANE Comparison of the physicochemical and physiological behaviour of natural inulin with its radioactive derivatives *Proc Soc exp Biol* (N Y 1963 *113* 584 588

DANIELSON, B G, J DELAHAYES and U SJOSTRAND, Technical and practical aspects of spectrometry for determination of radioactive isotopes in mixture *Acta Soc Med upsalien* 1966 173—195
DAVIES, J W and E C COCKING, Liquid scintillation counting of ^{14}C and ^{3}H samples using glassfiber or filterpaper discs *Biochim biophys Acta* (Amst) 1966 *115* 511—513
ENGBERG, A, A technique for repeated renal clearance measurements in undisturbed rats *Acta physiol scand* 1969 *75* 170—175
FALBRIARD A, R BUSSET and R ZENDER, Retention intrarenale d une faible fraction d inuline *Helv physiol pharmacol Acta* 1967 *25* 123—133
FRICK A, Reabsorption of inorganic phosphate in the rat kidney I Saturation of transport mechanism II Suppression of fractional phosphate reabsorption due to expansion of extra cellular fluid volume *Pflügers Arch ges Physiol* 1968 *304* 351—364
PHELPS, C F, The physical properties of inulin solutions *Biochem J* 1965 *95* 41—47
RENKIN, E M, Filtration, diffusion and molecular sieving through porous cellulose membranes *J gen Physiol* 1954 *22* 225—243
SNEDECOR G W, Statistical methods 5th Ed *Iowa State College Press* Ames Iowa 1956
THURAU, K, H VALTIN and J SCHNERMANN, Kidney *Ann Rev Physiol* 1968 *30* 441—524
exp Ther 1965 *149* 358—364

Acta physiol. scand. 1969. 76. 463—471
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On Adrenergic Influence on Gastric Motility in Chronically Vagotomized Cats

By

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Received 1 November 1968

Abstract

Jansson, G and B Lisander *On adrenergic influence on gastric motility in chronically vagotomized cats* Acta physiol scand 1969 76 463—471

Experiments were performed on chloralosed cats, which had been vagotomized 9—102 days

efferent low frequency stimulation of the splanchnic supply to the stomach promptly inhibited the stomach contractions After administration of atropine the above types of adrenergic activation elicited at most only very weak inhibitory response of the stomach and then only after a long latency —The data presented are compatible with the view that the adrenergic fibres to the stomach exert their *primary* action on stomach motility by inhibiting the intramural cholinergic ganglion cells rather than the smooth muscle cells, responsible for gastric myogenic tone, or by any "presynaptic inhibitory action" on the preganglionic vagal nerve endings

The interaction between the vagal excitatory fibres and the adrenergic fibres running to the stomach was studied in a recent investigation (Jansson and Martinson 1966) which showed that activation of the adrenergic supply to the stomach promptly inhibited vagally induced stomach motor waves In the absence of these waves adrenergic activation had at most very faint inhibitory effect on gastric myogenic tone" and then only after a comparatively long latency On the other hand this gastric tone level could be markedly suppressed by stimulation of the vagal relaxatory' fibres Furthermore gastric contraction induced by the muscarinic effect of acetylcholine was unaffected by such adrenergic activation as promptly inhibited a vagally induced contraction of similar magnitude

It would appear that the above findings can be best explained as an effect of the adrenergic supply to the stomach on cholinergic ganglia especially since neurohistological evidence of such a mechanism is available Norberg 1964 Jacobowitz 1965

Norberg 1967, personal communication) However, in *in vitro* studies on the stomach and *intestine* of guinea pigs and rabbits Gershon (1967) produced evidence suggesting a direct effect of adrenergic nerves on the gastro intestinal smooth muscle cells

Although, as mentioned, the studies by Norberg (1964), Jacobowitz (1965) and Jansson and Martinson (1966) suggest a ganglionic site of action of the main part of sympathetic outflow to the stomach, the site of action of the adrenergic nerve endings is still obscure They might exert their inhibitory action on any section of the vagal excitatory pathway below the cervical level, where also the possibility of a presynaptic inhibition of the preganglionic vagal fibres must be considered

Most if not all, efferent cholinergic fibres to the stomach run in the vagal nerves and after acute vagotomy the stomach seems devoid of contractions (Jansson and Martinson 1966) On the other hand, following chronic vagotomy in dogs Muren (1956) observed pronounced stomach motor waves, which could be abolished by hexamethonium or atropine Although the mechanisms responsible for these stomach motor waves were not studied further, the pharmacological characteristics mentioned above were sufficient to warrant the assumption of activity in parasympathetic post ganglionic neurons inducing the stomach contractions in these chronically vago tomized animals

The present investigation was undertaken to elucidate the adrenergic influence on the stomach contractions appearing after chronic vagotomy

Material and methods

12 cats weighing 3 0 to 5 5 kg were used They were vagotomized 9—102 days before the acute experiments

I Operative preparation for chronic vagotomy The operations were performed under nembutal anesthesia (30—40 mg/kg i p) The animals were intubated and artificial respiration was maintained by means of a respiration pump A longitudinal incision was made in the skin over the sternum which was exposed and divided along its entire length with a circular saw *Loose strings were placed around the esophagus slightly cranially to the diaphragm* All the vagal trunks were dissected free at this level and at least 2 cm of them were resected between ligatures The thorax was then carefully closed The animals recovered well and with one exception ate the day after the operation They were allowed food milk and water *ad libitum*

II Operative preparation for acute experiment Before the acute experiments the animals were deprived of food for 24—36 hrs They were then chloralosed (30 -80 mg/kg) and their stomach motility was recorded The two methods used for this purpose have been described in detail elsewhere (Jansson 1969 Jansson and Martinson 1965 1966) and will therefore only be briefly outlined here

a Balloon volume method In 4 experiments this method was used in the beginning of the [illegible]

diameter, and connected to a volume reservoir by a polyvinyl catheter The volume of the catheter situated in the balloon and the deflated balloon was about 16 ml which was included in the volumes given in the figures below The intragastric pressure was measured with "zero" reference to the pubic tubercle of the supine cat and was kept constant at 2 7 cm H_2O throughout the recording Occasionally the pressure was transiently raised to levels as high as 10 cm H_2O

b Direct intragastric volume method After midline incision of the abdominal wall the stomach lumen was shut off by a ligature passed underneath the vagus trunks and tied around the abdominal part of the esophagus while another ligature was placed around the duodenal

end of the stomach, where a widebore polyvinyl catheter was introduced into the gastric lumen The catheter was connected to a volume reservoir for continuous volumetric recordings

The sympathetic supply to the stomach was activated either direct by means of electric stimulation of the distal ends of the divided splanchnic nerves or reflexly by graded distension of a 10—15 cm long duodenal or jejuno-ileal loop, or by afferent electric stimulation of [illegible]

[illegible] impulse generator (Grass Stimulator S4) Stimulations of 1—32 imp/sec at a voltage of 2—8 and a pulse duration of 0 2—4 0 msec were used

To check that the chronic vagotomy was complete, the vagal nerves were dissected free at the cervical level and stimulated in the efferent direction with increasing voltage and duration of the impulses.

In 1 expt the spinal cord was transected between vertebrae C6 and C7 Spinal anesthesia was induced by subdural infusion of 1 5 ml of a 2 % lidocaine solution (Xylocain®, Astra) through a fine polyethylene catheter at the lumbar and thoracic levels

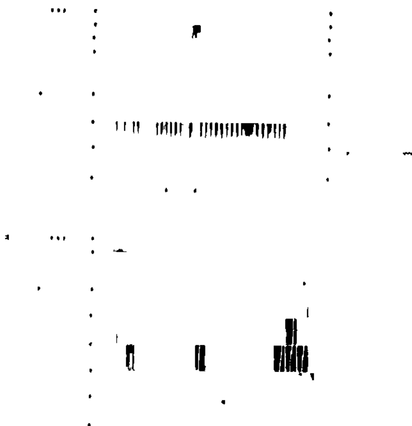
A femoral artery was cannulated to enable recording of the blood pressure by a mercury manometer [illegible] [illegible]pine sulphate Merck) was given intravenously in a [illegible] [illegible]ne (Ismelin®, CIBA) was given in a dose of 2 6 [illegible] was given by i m. injection of hydrocortisone [illegible]

Results

In acute experiments on 4 chloralose anesthetized animals, which had been vagotomized 16, 56 60 and 102 days earlier, gastric volume changes were recorded at constant intragastric pressure with the balloon volume method (Jansson 1969) All these experiments revealed regular and pronounced stomach contractions, which could be blocked by atropine in small doses (see Fig 1—3) The regular contrac tions were often powerful enough to empty the balloon even at intragastric pressures as high as 10 cm H_2O

Further analysis of the stomach contractions in the vagotomized animals required abdominal surgery Even during the midline skin incision a transient suppression of this stomach motility was recorded After incision of the abdominal wall and the surgical procedures necessary for adrenalectomy, isolation of the stomach and of the intestinal loops the stomach contractions regularly disappeared for at least two hours (see Fig 1 and 3) This suppression of the motility was evidently due to activity of the adrenergic nerve supply to the stomach since it could be completely blocked by guanethidine Fig 3) by spinal anesthesia (Fig 2) or by section of the splanchnic nerves supplying the stomach Fig 1 On the other hand in the cats where the splanchnic nerves had been divided the suppression of the motor waves could be induced again by efferent stimulation of the divided splanchnic nerves (Fig 1) The abdominal incision thus evidently produced a marked reflex tonic discharge in the splanchnic nerves relayed *via* spinal connections and exerting an adrenergic inhibitory effect suppressing the stomach contractions Moreover if it is assumed that these stomach motor wave in chronically vagotomized animals are dependent on neurogenic mechanisms these mechanisms reside in the stomach wall

As mentioned above all the contractions of the stomach in the anesthetized disappeared for at least two hours after abdominal surgery When one of the [illegible]nic nerves was then cut and its efferent end stimulated, it produced at



... in acute experiment on ... 7 cm H_2O ... control of completeness of ... and increasing duration of impulses Note transient suppression of the stomach motility on stimulation with 2 msec impulses.

Upper right part Motility suppressed after abdominal surgery necessary for adrenalectomy and preparation of right splanchnic nerves. Note weak inhibitory gastric responses on efferent stimulation of right splanchnic nerves (2 msec 8 V)

Lower part Return of stomach contractions after division of all detectable splanchnic nerves. ... stomach

faint increase in volume provided the other splanchnic connections were still intact Neither did intestinal distension nor mesenteric afferent stimulation result in any prompt or significant decrease in gastric tone under these conditions In other words the presence of contractions in the stomach was necessary for the elicitation of pronounced inhibitory responses by sympathetic activation This is obvious from the experiments illustrated in Fig 1 and 4 The upper part of Fig 1 shows the complete

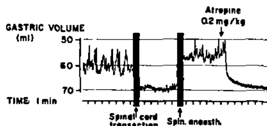


Fig 2 Cat 3 5 kg Chronic vagotomy 56 days before acute experiment Stomach motility, which was suppressed by spinal cord transection between vertebrae C6—C7 (middle part, actual recording about 40 min after transection) Spinal anesthesia blocked this suppression and contractions returned

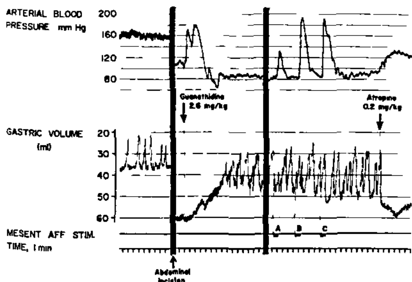


Fig 3 Cat 3 9 kg Records of gastric motility with balloon volume method in acute experiment 60 days after vagotomy

Before laparotomy there were marked contractions (left panel) which disappeared on abdominal incision (left part of middle section)

Administration of guanethidine was followed by return of stomach contractions

Right part Electric stimulation of mesenteric afferent nerves A 4 imp/sec 1 msec 3 V B 4 imp/sec 5 msec 5 V C 20 imp/sec 5 msec 5 V The gastric motor waves were blocked by atropine (The animal was not adrenalectomized)

disappearance of the stomach contractions after abdominal surgery In this situation stimulation of the splanchnic nerve elicited no significant (4 imp/sec) or only a small (8 imp/sec) inhibitory response in the stomach and then with a long latency On the other hand after bilateral division of the splanchnic nerves (lower part of Fig 1) the stomach contractions returned and they could be completely and promptly inhibited by even l...

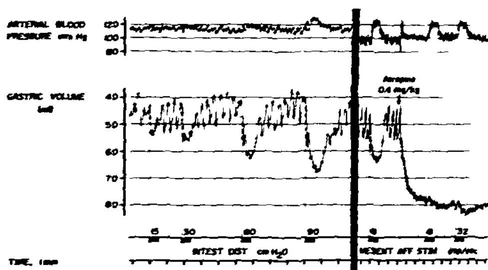


Fig. 4. Cat 3.8 kg. Vagotomized 48 days before acute experiment. Effect of graded duodenal distension (left part) and electric stimulation of mesenteric afferents upon stomach contractions. Note faint inhibitory reflex responses with long latency after atropine. Time from abdominal preparation to actual recording about 3 hrs. Mesenteric afferent stimulation 3 msec, 5 V.

these inhibitory responses was as short as 3—4 sec. compared with a latency of about 20—30 sec for the weak inhibitory responses obtained by high frequency splanchnic stimulation when the contractions were absent.

In some experiments the stomach contractions returned 2—4 hrs after the abdominal preparation even when the splanchnic nerves were left intact. In these experiments the intestino-gastric inhibitory reflex (cf. Jansson and Martinson 1966) was studied. Graded intestinal distension (Fig. 4, left part) and electric stimulation of mesenteric nerves in such an animal (Fig. 4, right part) promptly inhibited gastric motility. These inhibitory responses could be blocked by guanethidine.

Atropine completely blocked the gastric contractions and then the adrenergic suppression of the prevailing gastric tone was also more or less abolished. When present this suppression was quite small and slow in onset. Significant adrenergic inhibitory responses could then usually be obtained only on efferent splanchnic stimulation with high frequencies (8 or 16 imp/sec) (Fig. 1).

No augmentative stomach motor responses were ever recorded when the completeness of the chronic vagotomy was checked by bilateral electric stimulation of the distal ends of the cut cervical vagus with stepwise increasing impulse duration to

activate any residual excitatory vagal fibres running to the stomach On the other hand, in two experiments intense vagal stimulation inhibited the prevailing stomach contractions and produced a considerable simultaneous fall in blood pressure (Fig 1) This inhibition was presumably adrenergic, since it could be blocked by guanethidine but no further analysis of these occasional findings was made

Discussion

Investigating the pronounced stomach motor waves after chronic vagotomy Muren (1956) observed that the contractions could be blocked by atropine and hexamethonium both in unanesthetized and anesthetized dogs This suggests that nervous activity involving the parasympathetic postganglionic neurons was responsible for the increased motility Hexamethonium and atropine in the concentrations used seem not to affect "myogenic tone" (*cf* Martinson 1965, Jansson and Martinson 1966) In the present study it was shown that this type of stomach contractions persisted during spinal anesthesia and after division of the splanchnic supply to the stomach The observations indicate that an inherent' nervous activity of intramural parasympathetic neurons of the stomach are responsible for this motility Local intramural cholinergic reflex connections may perhaps, become sensitized after chronic vagotomy and act 'spontaneously' to a far greater extent than in the acutely vagotomized stomach and thereby elicit the characteristic stomach contractions No such motor waves occur after acute vagotomy (Jansson and Martinson 1966), not even when splanchnic nerve connections are divided or when guanethidine has been given (Jansson unpublished observations)

It was further studied whether these apparently intramurally induced stomach contractions were under adrenergic control It was found (Fig 1 and 4) that both a direct efferent splanchnic stimulation even at low frequencies and a reflex activation of these connections by intestinal distension or mesenteric afferent nerve stimulation markedly and promptly inhibited such motility The adrenergic nervous activity seemed preferentially to antagonize the stomach contractions which were in turn dependent on cholinergic nervous activity in the chronically vagotomized stomach In fact the inhibition of stomach motility by the adrenergic nerves could be clearly shown only in the presence of this type of motility In the absence of the contractions similar adrenergic activation had at most a weak inhibitory effect on gastric tone and then with a comparatively long latency Thus on efferent direct stimulation of the splanchnic nerves with supraphysiological frequencies (especially with 16 or 32 imp/sec) these slow inhibitory responses could be elicited an observation also suggesting the existence of a tone that could be affected The probable explanation of the inhibition produced by supraphysiological sympathetic stimulation is an overflow of the adrenergic transmitter substance from *e g* vasoconstrictor nerve endings and adrenergic nerve endings involved in motility regulation (*cf* Brown 1960) In contrast to these weak inhibitory responses the stomach contractio the chronically vagotomized animals could be completely and promptly inhibi

adrenergic stimulation at a comparatively low frequency. Therefore demonstration of direct adrenergic inhibitory effects on the stomach required the presence of cholinergic activity or, in other words, a "nervous cholinergic tone".

The fact that the adrenergic inhibitory effects in the present experiments were so clearly demonstrated only in the presence of the cholinergic nervous activity mentioned above, lends further support to the view that the adrenergic control of gastric motility operates at least mainly by antagonizing the cholinergic activity in the stomach. Neuro-histochemical studies strongly suggest that the terminals of adrenergic nerves other than vasomotor fibres are arranged around intramural ganglionic cells in the stomach and intestinal wall (Norberg 1964, Jacobowitz 1965, Norberg 1967, personal communication). Jansson and Martinson (1966) explained their findings as a result of an adrenergic inhibition of intramural ganglionic cells involved in the vagal excitatory pathway to the smooth muscles of the stomach. The responses described by them showed characteristics essentially identical with the adrenergic inhibition described in the present study. Gershon (1967) studied the site of action for sympathetic stimuli in the gastro-intestinal tract. His results were taken as evidence of a direct action of sympathetic nerve stimulation on the smooth muscles. This conclusion was based on *in vitro* experiments on rodents and, as far as the stomach was concerned, mainly on the observation that perivascular sympathetic stimulation and noradrenaline elicited inhibitory responses in the presence of hyoscine.

It should here be stressed that different sites of action of the adrenergic nerve terminals in the stomach may well exist (*e.g.* smooth muscles and ganglia) and that they may differ in relative importance in different species. Demonstration of the subtle nervous mechanism involving a ganglionic site of action of adrenergic nerve terminals seems to require an *in vivo* preparation with intact blood supply and 'normal' internal environment. In such experiments the responses should be closely studied for any quantitative or qualitative differencies. The present findings are well compatible with the hypothesis that the adrenergic fibres act primarily upon cholinergic ganglionic cells involved in the excitatory influence on the gastric smooth muscle cells, but do not exclude the possibility of adrenergic nerve fibres with direct action on gastric smooth muscles, nor do the results obtained by Gershon (1967) exclude a cholinergic ganglionic site of action of the adrenergic nerves to the stomach. It should be stressed, however, that the adrenergic nerves virtually invest and terminate around intramural cholinergic ganglion cells and do not end in the muscle layers of intestine (Norberg 1964) and the stomach (Jacobowitz 1965, Norberg 1967, personal communication). Furthermore, gastric smooth muscle activity produced by the *direct* muscarinic effect of infused acetylcholine is unaffected by such sympathetic stimulation as promptly inhibits vagally induced contractions of similar magnitude (Jansson and Martinson 1966).

It is known that abdominal surgery markedly affects gastro-intestinal motility (*e.g.* Cannon and Murphy 1906). From the present experiments, illustrated in Fig. 1 and 3, it is obvious that abdominal surgery activates adrenergic reflexes which could completely suppress the stomach contractions for a considerable time. Thus under

such conditions it may be impossible to demonstrate the existence of an inhibitory response on adrenergic stimulation simply because a pronounced inhibition is already present owing to tonic reflex activity

The effect of spinal anesthesia on the stomach contractions in a chronically vagotomized cat was studied in the experiment illustrated in Fig 2 In the same experiment suppression of gastric motility was observed after spinal cord transection but this finding was not made the subject of further investigation This suppression was in all probability due to increased activity in adrenergic reflex arcs and was thus in conformity with the finding by Johansson Jonsson and Ljung (1968) in their study of the supraspinal control of the intestino intestinal inhibitory reflex

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References

Brown G L Release of sympathetic transmitter by nerve stimulation *Ciba Found Symp on Adrenergic Mechanisms* J & A Churchill Ltd London 1960 pp 116—124

Cannon W B and F T Murphy The movements of the stomach and intestines in some surgical conditions *Ann Surg* 1906 *43* 512—536

Gershon M D Inhibition of gastrointestinal movement by sympathetic nerve stimulation the site of action *J Physiol* (Lond) 1967 *189* 317—327

Jacobowitz D Histochemical studies of the autonomic innervation of the gut *J Pharmacol Exp Therap* 1965 *149* 358—364

Jansson G Vago-vagal reflex relaxation of the stomach in the cat *Acta physiol scand* 1969 *75* 245—252

Jansson G and J Martinson Some quantitative considerations on vagally induced relaxation of the gastric smooth muscle in the cat *Acta physiol scand* 1965 *63* 351—357

Jansson G and J Martinson Studies on the ganglionic site of action of sym [illegible] flow to the stomach *Acta physiol scand* 1966 *68* 184 192

Johansson B O Jonsson and B Ljung Tonic supraspinal mechanisms influencing the intestino-intestinal inhibitory reflex *Acta physiol scand* 1968 *72* 200—201

Martinson J Vagal relaxation of the stomach [illegible] of the transmission mechanism *Acta physiol sc* [illegible]

Muren A Gastric motility after vagotomy in do [illegible]

Norberg K A Adrenergic innervation of the i [illegible] scopy *Int J Neuropharmacol* 1964 *3* 3 [illegible] 382

Norberg K A Personal communication 1967

Acta physiol scand 1969. 76. 472—484

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Effects of Thermal Treatment on Mitochondria of Brain, Liver and Ascites Cells

By

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Received 12 November 1968

Abstract

Christiansen, E N and E Kvamme *Effects of thermal treatment on mitochondria of brain liver and ascites cells* Acta physiol scand 1969 76 472—481

The oxidative phosphorylation and respiratory control of mouse brain liver and Ehrlich ascites tumour cells have been studied after preincubating the mitochondria *in vitro* at temperatures up to 45° Respiration phosphorylation and respiratory control were measured with malate [illegible]

phosphorylation were determined The results indicate that electro[illegible] cytochrome c region is more heat sensitive than that of the cytochrome c-oxygen region Moreover both this inhibition of electron transport loss of respiratory control and uncoupling *of phosphorylation are likely to be primary effects of heat treatment* *Leakage of endogenous* cytochrome c to the surrounding medium has been demonstrated indicating membrane damage as an early effect of heat treatment The investigated tissues show considerable variations in heat sensitivity of oxidative phosphorylation brain mitochondria being the most heat resistant Oxidative phosphorylation of mitochondria prepared from heat treated *intact* ascites cells is more heat resistant than that of *in vitro* heat treated mitochondria as higher temperatures are required to produce similar effects

The effects of mild thermal treatment have previously been studied in the Keilin Hartree heart muscle preparation (Morris and King 1962) It was found that NADH→oxygen and NADH→cytochrome c activities were most sensitive to thermal inactivation whereas cytochrome oxidase activity was almost unaffected Furthermore ageing has been shown to inhibit the various processes involved in oxidative phosphorylation unequally (Redfearn and Dixon 1961 Rochman Lathe and Levell 1967, Slater 1949) Thus it seemed possible to make use of mild heat treatment as a method to influence energy metabolism selectively even in intact cells

Mouse brain liver and ascites tumour cells were chosen for the studies because of the great differences in oxidative metabolism between these tissues Oxidative phos

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phorylation of ascites cells is also of particular interest due to the yet unexplained Crabtree effect (Coe 1966, Gordon, Ernster and Dallner 1967, Nigam 1966 Overgaard Hansen 1965)

In the present communication it is reported that brain mitochondria are considerably more heat resistant than the other tissues investigated a fact which confirms that there exist variations in heat sensitivity between tissues of different origin Moreover, electron transport in the succinate cytochrome c region is more heat sensitive than that of the terminal segment, and coupling of phosphorylation is generally labile to thermal treatment In addition, evidence will be presented that the cytochrome c content of mitochondria decreases upon heat treatment

Materials and methods

Reagents

N,N,N',N', tetramethyl 1 4 phenylenediamine (TMPD) was obtained from Fluka A G The phenazine methosulphate (PMS), ATP, hexokinase cytochrome c, rotenone and antimycin A used were products of the Sigma Corporation The other reagents were of highest purity, analytical grade

Ascites tumour cells

Ehrlich ascites tumour cells were obtained from Dr L Revesz Karolinska Institutet Stock

both sexes ac

twice in saline

ished cells were

4 0 04 M, tris

Preparation of mitochondria

0 25 M and EDTA

v/v) 13 ml of

ml of Ballotini

a 0 5 cm thick

igned and made

mpletely broken

the procedure The cell suspension ... ice before the homogenization and the temperature of the homogenate at the end was +2°

Mouse liver and brain were homogenized in 0 30 M sucrose containing EDTA (liver 0 5 mg/ml brain 0 2 mg/ml) and using a Potter Elvehjem homogenizer The homogenates were first centrifuged for 5 min at 450 × *g* and the supernatants then centrifuged for 10 min at 20 000 × *g* in a Sorvall centrifuge SS4 rotor No SS34 The mitochondrial precipitates were washed twice

Heat treatment

Brain and

MgCl 0

tris-HCl p

mg/ml)

mitochondria except that sucrose was replaced by mannitol 0 12 M and the EDTA concentration changed to 0 1 mg/ml *Heat treatment of whole cells or mitochondria was performed* either for 10 min at pre set temperatures ± 0 1° (up to 45° or for varying time intervals at 45° in a thermostatic controlled water bath The samples were cooled in ice before the measurement of oxygen uptake and phosphorylation

Oxygen uptake

The oxygen uptake was measured at 30 by standard Warburg technique using 15 ml F and 0 2 ml 10 % KOH on a roll of filter paper in the center well The total volu incubation mixture was 2 ml and readings were taken every 10 min The reaction was by the addition of perchloric acid 0 3 M final concentration), and the precipitat d with KOH

Oxidative phosphorylation

... sucrose 0 15 M, $MgCl_2$ 0 006 M, KCl 0 04 M potassium phosphate pH 7 4 (liver 0 03 M brain 0 04 M), tris HCl pH 7 4, (liver, 0 007 M, brain, 0 02 M) and EDTA liver, 0 2 mg/ml brain 0 01 mg/ml) The sucrose of the medium used for brain was replaced by mannitol (0 10 M) and the EDTA concentration changed to 0 05 mg/ml in the medium for ascites cell mitochondria. The composition of the media was such as to ensure maximal oxidative phosphorylation The medium for brain mitochondria was essentially similar to that used by *Balázs (1965)* The mitochondria suspension was added in a total volume of 2 ml Oxidative phosphorylation at the sites I+II+III including substrate linked phosphorylation was measured in the presence of malate, 0 012 M, pyruvate, 0 006 M and NAD, 0 005 M NAD was added to ensure a sufficient concentration of this cofactor in case heat treatment in some way would affect the endogenous supply The ... lation of oxygen uptake of untreated mitochondria ... +III was determined in the presence of succinate, C... electron transport observed in the absence of pho... termined *according to Lee Sottocasa and Ernster (1967)* Thus P/2e at site II was assayed by measuring both the reduction of ferricyanide (0 0025 M) by succinate in the presence of rotenone ($0\,3 \times 10^{-6}$ M) and KCN (0 001 M), as well as the phosphorylation coupled to this reduction at 25° The reduction of ferricyanide was ...
and corrected for that value in the pre...
phosphorylation was corrected for any...
and Racker 1966) Th...
is important in view of ...
mitochondria as shown ...
TMPD $0\,4 \times 10^{-3}$ M a...
µg (Schatz *et al* 1966) The results were corrected for phosphate and oxygen uptake in the same assay system in which cyanide was added The presence of rotenone inhibited oxygen uptake with malate plus pyruvate as substrate However no inhibition of ferricyanide reduction by succinate or oxygen uptake in the cytochrome c—oxygen region and the coupled phosphorylation (site II III) could be demonstrated Thus the addition of rotenone was unnecessary for these determinations This is in accordance with the low oxygen uptake in intact mitochondria without added substrate The oxidative phosphorylation at the sites I+III including substrate linked phosphorylation was assayed by the addition of TMPD antimycin A and malate plus pyruvate in the above mentioned concentrations using oxygen as ultimate electron acceptor (Lee Nordenbrand and Ernster 1965 Packer and Mustafa 1966)

Phosphate uptake was determined according to Conover Prairie and Racker (1963) Their method is a slight modification of that of Lindberg and Ernster (1956) Inorganic phosphate was measured following extraction by isobutane/benzene (Martin and Doty 1949 and a Packard liquid scintillation counter used for the counting of radioactive phosphate in Bray's solution (Bray 1960 Both the isobutane/benzene phase and the aqueous phase were counted

Results

Mitochondrial oxygen and phosphate uptake were both sensitive to heat treatment with succinate and malate plus pyruvate as substrates particularly in ascites cells and liver Fig 1 demonstrates the percentage inhibition of oxygen uptake (1a) and phosphorylation (1b) following heat treatment Mild heat treatment (temperatures below 41°) of liver and ascites cell mitochondria affected phosphorylation slightly more than oxygen uptake the P/O ratios remaining almost constant (Table 1 This was particularly striking when using malate plus pyruvate (B as substrates The initially rather stable P/O ratios (in spite of declining oxygen and phosphate uptake) can be explained in two ways It may be an all or none effect i e oxidative phosphorylation of some of the mitochondria ceased completely and no inhibition of that of the remaining ones occurred Alternatively the phenomenon might be ex

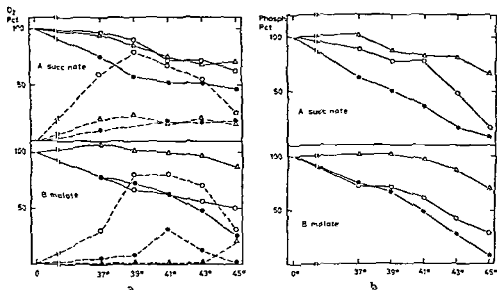


Fig 1 The per cent reduction in oxygen uptake (1a) and phosphorylation (1b) following heat treatment of the mitochondria The mitochondria were heat treated for 10 min at 37°—45 and cooled in ice The composition of the incubation medium for the assay was the same as in Table I The assay was performed at 30

100 % indicates µatoms of oxygen or phosphate taken up per mg protein per 30 min in mitochon dria
liver
Abso
ascite

△ brain mitochondria
● ascites cell mitochondria

NAD 0 003 M

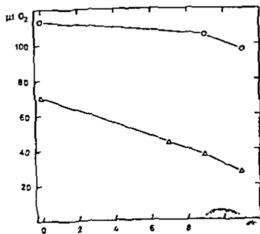


Fig 2 The effect of heat treatment on ascites cell mitochondria assayed with succinate and phenazine methosulfate The experimental conditions were the same as in Table I, except that the mitochondria were heat treated for 7—11 min at 45 as indicated
△ added succinate 0 0125 M
○ added succinate 0 0125 M phenazine methosulfate 1 mg CaCl 0 00125 M and KCN 0 001 M

TABLE I *The effect of heat treatment on isolated brain, liver and ascites cell mitochondria*

The mitochondria were prepared and heat treated for 10 min at temperatures from 37°—45 as described in the text The assay was performed at 30° for 30 min in a medium of the following composition (total volume 2 ml) Bovine albumin 2 mg/ml, cytochrome c, 2 5 × 10 ⁴M, MgCl₂ 0 006 M, KCl, 0 04 M, potassium phosphate pH 7 4, 0 04 M, tris HCl pH 7 4, 0 02 M, [³²P] 15 μC, hexokinase, 0 2 mg/ml, glucose, 0 05 M and ATP, 0 8 × 10 ³M In addition the incubation media contained for liver mitochondria sucrose, 0 15 M, EDTA, 0 2 mg/ml, for brain mitochondria sucrose, 0 15 M EDTA 0 1 mg/ml, for ascites cell mitochondria mannitol, 0 10 M, EDTA 0 05 mg/ml Each experiment was performed 3 times R C respiratory control, *i e* the ratio of oxygen uptake in the presence of hexokinase, glucose and ATP to that in the absence Added malate, 0 0120 M pyruvate 0 006 M and NAD, 0 005 M Added succinate, 0 0125 M

Substrate	Cell type		0°	37°	39°	41°	43°	45°
Malate +	Brain	P/O	2 6	2 5	2 7	2 5	2 4	2 1
Pyruvate		R C	10 7	13 3	11 7		10 4	6 3
	Liver	P/O	2 6	2 8	2 3	2 4	2 0	1 3
		R C	3 3	2 6	1 4	1 5	1 4	1 4
	Ascites	P/O	3 0	2 7	2 3	2 0	0 9	0 8
	cells	R C	3 8	2 5	2 3	1 6	1 4	1 6
cinate	Brain	P O	1 8	2 0	2 0	2 0	2 1	1 8
		R C	1 8	1 7	1 4	1 4	1 2	1 1
	Liver	P O	1 8	1 4	1 5	1 2	1 0	0 7
		R C	2 0	1 3	1 2	1 1	0 9	0 9
	Ascites	P O	2 2	1 9	1 6	1 4	1 2	0 3
	cells	R C	2 0	1 2	1 1	1 0	1 0	1 0

plained by a partial inhibition (with no uncoupling) either of the electron transport or of the phosphorylation of all the mitochondria If phosphorylation was initially inhibited *e g* by a mechanism similar to that of oligomycin uncouplers like 2 4-dinitrophenol (DNP) ought to stimulate oxygen uptake Experiments carried out to elucidate this point failed to disclose any stimulation by DNP under these conditions

As shown in Table I respiratory control generally declined more rapidly upon heat treatment than did P/O ratios Since respiratory control is measured as the ratio of state III respiration to that of state IV Chance and Williams 1956 a fall in respiratory control could be due to either a decrease in state III respiration or an increase in state IV respiration State IV respiration (dotted line in Fig 1a) is measured by oxygen uptake with no phosphate acceptor system added (non phosphorylating electron transport (Chance *et al* 1956) It is indicated that the percentage decrease in state III respiration of ascites cell mitochondria is higher than the corresponding increase in state IV respiration In brain mitochondria state III and state IV respiration seem to contribute about equally to the drop in respiratory

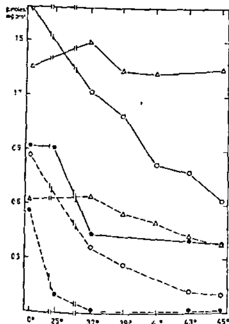


Fig 3

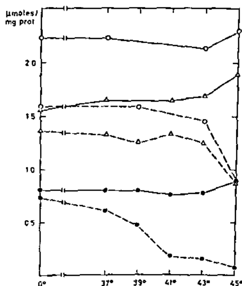


Fig 4

Fig 3 The eff ...

... ferricyanide reduction was followed spectrophotometrically at 420 mµ (25°) The reduction of ferricyanide was corrected for that in the presence of antimycin A (1 µg/mg protein) and the coupled phosphorylation for that in the absence of ferricyanide

- ○ liver mitochondria
- △ brain mitochondria
- ● ascites cell mitochondria
- — ferricyanide reduction
- -- phosphorylation

... The mitochondria were ... r 10 min at temperatures ... is the same as in Table I ... otenone 0 3 × 10⁻⁶ M and ... in at 37°

- ○ liver mitochondria
- △ brain mitochondria
- ● ascites cell mitochondria
- — oxygen uptake
- -- phosphorylation

control, but in liver mitochondria the decrease in respiratory control is mostly due to an increased state IV respiration

Stronger heat treatment (temperatures in the range 41°—45°) generally ... phosphorylation considerably more than oxygen uptake which indicates ... of phosphorylation

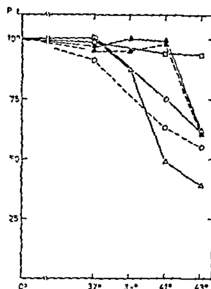


Fig 9 The per cent reduction in mitochondrial oxygen uptake and phosphorylation following heat treatment of intact ascites cells The whole cells were heat treated for 10 min at temperatures in the range 37°—45° and the mitochondria prepared as described in the text Oxygen uptake was measured at 30 and ferricyanide reduction at 25° The ferricyanide reduction was corrected for that in the presence of antimycin (1 µg mg protein) The composition of the incubation medium was the same as in Table I

▲ added malate 0 0120 M pyruvate 0 006 M and NAD 0 005 M
○ added succinate 0 0125 M
△ added ferricyanide 0 0025 M succinate 0 0125 M rotenone 0 3 × 10 ⁶ M and KCN 1 × 10 ³ M (see text) Ferricyanide reduction was measured at 420 mµ
□ assay of phosphorylation site III Ascorbate pH 7 4 0 010 M TMPD 0 4 × 10 ³ M rotenone 0 3 × 10 ⁶ M and antimycin 1 µg
— oxygen uptake or ferricyanide reduction
--- phosphorylation

tochondria. Insignificant phosphate uptake could be measured in ascites cell mitochondria upon heat treatment at temperatures in the range 25°—37° Phosphate uptake in brain mitochondria was however not lowered under these conditions (Fig 3 5) Furthermore in contrast to brain mitochondria respiratory control at phosphorylation site II was lost and P/O ratios markedly decreased in ascites cell and liver mitochondria (Kvamme and Christiansen 1967)

Oxygen uptake coupled to phosphorylation at site III was assayed using ascorbic acid as electron donor for TMPD and oxygen as ultimate electron acceptor in the presence of rotenone and antimycin (Schatz et al 1966) (Fig 4) Under these conditions oxygen uptake was quite heat resistant in mitochondria from ascites cells liver and brain whereas phosphate uptake (Fig 4) and P/O ratios (Fig 5) in ascites cell mitochondria were considerably more heat sensitive than those of liver and brain mitochondria This indicates that electron transport in the succinate—cytochrome c region are particularly heat sensitive in ascites cells and liver

Oxidation and coupled phosphorylation at the sites I + III of ascites cells were assayed using malate as hydrogen donor for NAD antimycin to block site II TMPD as a bypass for this block and oxygen as ultimate electron acceptor (Lee et al 1965) (Fig 6) Since oxygen and phosphate uptake decreased rapidly under these conditions and electron transport at site III was shown to be quite heat resistant electron transport at site I seemed likely to be heat sensitive Fig 3 4 and 5 also indicate that phosphorylation at site III in ascites cell and liver mitochondria was less affected by thermal treatment than phosphorylation at site II However brain mitochondria did not show any such difference

Mitochondrial oxygen uptake particularly of ascites cells was stimulated by the addition of cytochrome c and the stimulation became more pronounced in heat

treated mitochondria (Fig 7) When oxygen uptake in the cytochrome c—oxygen region was assayed the effect of cytochrome c addition was striking The data indicate that the endogenous supply of cytochrome c was rapidly depleted by heat treatment Since the heat treatment did not destroy an equally buffered suspension of pure cytochrome c the endogenous supply of cytochrome c was presumably lost to the medium through a heat damaged mitochondrial membrane In support of this view the extinction at 410 mμ of the suspending medium was found to increase following heat treatment (Fig 8a) Furthermore the difference spectrum of the suspending medium from heat treated mitochondria (45°, 10 min) against that from non heat treated mitochondria showed peaks at 415 and 545—550 mμ upon reduction with the dithionite (Fig 8b)

The oxidative phosphorylation of mitochondria prepared from intact ascites cells exposed to heat treatment is shown in Fig 9 Under these conditions oxidative phosphorylation was somewhat more heat resistant than that of heat treated isolated mitochondria (*Cf* Fig 1a 1b and 9) The data indicate that electron transport in the succinate (or NADH)—cytochrome c region is heat sensitive in contrast to that of the cytochrome c—oxygen region also in heat treated intact cells

Endogenous respiration of intact ascites cells was greatly inhibited by heat treatment whereas glycolysis and respiration in the presence of glucose (0 03 M) were little affected In other words the Crabtree effect was reduced and almost disappeared by heat treatment (Kvamme *et al* 1967) This is in accordance with the general hypothesis that the Crabtree effect is due to inhibition of oxidative phosphorylation (Racker 1956) It is also in agreement with the great heat resistance of non phosphorylating electron transport in isolated mitochondria

Discussion

It is well known that the three phosphorylation sites might be selectively inhibited by different inhibitors (Slater 1966) Heat is another means of affecting oxidative phosphorylation and this inhibition might also be possible to produce *in vivo* The ageing phenomenon of mitochondria is related to the effect of mild heat treatment However the conditions for ageing have been different in various investigations with respect to temperature and time used The variation of these parameters may affect different regions of the respiratory chain. No direct comparison can thus be made between ageing and the heat treatment used in this work It has been suggested that the cytochrome b segment of heart mitochondria is the most heat sensitive part of the respiratory chain (Morris *et al* 1962) which conforms with the present findings Since succinate dehydrogenase is quite heat resistant when assayed by the phenazine methosulphate method it seems probable that the first carriers to receive electrons are heat resistant as they are believed to be associated with the enzyme itself Furthermore as the electron transport between succinate and cytochrome c is more l sensitive than that of the terminal segment the most heat sensitive part is l be localized to the cytochrome b segment.

If cytochrome c is not supplemented to the medium, the respiration declines due to loss of cytochrome c from the mitochondria. This indicates that the structural membrane is affected by heat making the mitochondria leaky for cytochrome c. Damage of the mitochondrial structure might also liberate lipid peroxides and free fatty acids derived from structurally bound lipids, whereby the uncoupling effect might be explained (Wojtczak and Lehninger 1961).

Various chemical and physical inhibitors (e.g. irradiation, Clark 1967) produce a stronger effect on respiratory control than that on P/O ratios. Since the P/O ratio is an expression of the overall stoichiometry of oxidative phosphorylation, the respiratory control may be a better parameter for the intactness of the phosphorylating system. It is indicated that the fall in respiratory control in ascites cells is caused by a decreased state III respiration which may in turn be explained by an inhibited electron transfer or by a decreased production or availability of reducing equivalents. In liver the fall in respiratory control is largely caused by an increased state IV respiration which may be correlated with an increased ATP-ase activity. It is reported (Chance 1959) that the respiratory control is affected by even slight mitochondrial membrane damage. The hypothesis that loss of respiratory control is caused by membrane damage, is supported by the finding of cytochrome c leakage.

Concerning the differences in heat sensitivity of ascites cell, liver and brain mitochondria, it should be noted that media of different composition were used for thermal treatment of these tissues. The media selected were previously found to give optimal oxidative phosphorylation for the tissues in question, based on studies reported in the literature. Control experiments interchanging the media used for brain and ascites cell mitochondria did not make ascites cell mitochondria more heat resistant and vice versa. However, on lowering the EDTA concentration (to 0.1 mg/ml) and increasing the tris concentration, liver mitochondria were more easily uncoupled, but electron transport was now almost insensitive to heat treatment. In this connection it is of interest that Good *et al.* (1966) have described an inhibitory effect of tris buffer on oxidative phosphorylation.

The demonstrated differences in heat sensitivity of various tissues resemble the well established differences in radiosensitivity, which might also reflect variations in mitochondrial structure or enzyme systems. Of particular interest is the great heat sensitivity *of ascites tumour cells in contrast to that of brain*.

If enzymes were liberated from lysosomes which contaminate the mitochondrial preparations (de Duve *et al.* 1955) they might contribute to the measured heat effect on isolated mitochondria. However, a similar mechanism would also be expected to operate by heat treatment of intact cells, since oxidative phosphorylation is affected in the same way as in isolated mitochondria. The main difference seems to be that mitochondria of intact cells are somewhat protected by the surrounding cell material so that a higher temperature is required to give a specific inhibition.

The effects of thermal treatment involve certain physiological and pathological aspects. The lability of oxidative phosphorylation of tissues such as liver to temperatures in the range 41°—45° may explain the sensitivity of mammals to a raise

in body temperature above a certain critical level within this range It is possible *that an irreversible uncoupling of phosphorylation occurs in the liver under such* circumstances

This investigation was supported by grants from the Norwegian Society for fighting Cancer and Grosserer N A Stangs Legat til Kreftsykdommers Bekjempelse Preliminary experiments were aided by Anders Jahres fond til Vitenskapens fremme The authors are indebted to Professor L. Ernster Institute of Biochemistry University of Stockholm Sweden, for valuable suggestions and criticism during the preparation of this manuscript The skilled assistance of i strument maker B Andersen is gratefully acknowledged

References

BALAZS R Control of glutamate oxidation in brain and liver mitochondrial systems *Biochem J* 1965 95 497—508

BERNATH P and T P SINGER *Succinic dehydrogenase* In Methods in Enzymology, ed by S P Colowick and N O Kaplan Academic Press New York 1962 Vol 5 597—614

BRAY, G A A simple efficient liquid scintillator for counting aqueous olutions in a liquid scintillation counter *Analyt Biochem* 1960 *1* 279—285

CHANCE B *Quantitative aspects of the control of oxygen utilization* In Ciba Foundation Symposium on the regulation of cell metabolism ed by G E W Wolstenholme and C M O Connor J & A Churchill Ltd London 1959 91—129

CHANCE B and G R WILLIAMS The respiratory chain and oxidative phosphorylation *Advanc Enzymol* 1956 *17* 65—134

CLARK J B, Effect of irradiation in vitro on mitochondrial metabolism Oxidation of citric acid cycle substrates *Europ J Biochem* 1967 *2* 19—25

C[illegible]

C[illegible]

DE DUVE C B C PRESSMAN R GIANETTO R WATTIAUX and F APPELMANS Tissue fractionation studies 6 Intracellular distribution patterns of enzymes in rat liver tissue *Biochem J* 1955 *60* 604—617

GOOD N E, G D WINGET [illegible] and R M M SINGH Hydrogen ion buffers for [illegible] 467—477

GORDON E E L ERNSTER [illegible] en transport in Ehrlich ascites tumor cells *Cance*[illegible]

KLEIN G and L REVESZ Quantitative studies on the multiplication of neoplastic cells in vivo I Growth curves of the Ehrlich and MC1M ascites tumour *J nat Cancer Inst* 1953 *14* 229—277

KVAMME E Glycolysis and respiration in Ehrlich ascites tumour cells I Phosphate metabolism in relation to glycolysis and the Crabtree effect *Acta physiol scand* 1958 *42* 204—218

KVAMME E and E N CHRISTIANSEN Unpublished observation 1967

LEE C P K NORDENBRAND and L ERNSTER *Electron transport and oxidative phosphorylation in the cytochrome b c region of the respiratory chain as studied with the tetramethylphenylenediamine shunt over the antimycin a sensitive site* In Proc Intern Symp Oxidases Related Redox Systems Amherst Mass 1964 Wiley New York 1965 960—982

LEE C P G L SOTTOCASA and L ERNSTER *Use of artificial electron acceptors for abbreviated phosphorylating electron transport Flavin cytochrome C* In Methods in Enzymology ed by S P Colowick and N O Kaplan Academic Press New York 1967 Vol *10* 33—3

LINDBERG O and L ERNSTER *Determination of organic phosphorus compounds by phosphate analysis* In Methods of Biochemical Analysis ed by D Glick Interscience Publishers Inc New York 1956 Vol *3* 1 22

M[illegible] inorganic phosphate (Modification [illegible]

M[illegible] ation of the heart muscle prepa[illegible]

NIGAM V N A study of the Crabtree effect in Novikoff ascites-hepatoma cells *Biochem J* 1966 *99* 413—418

OVERGAARD-HANSEN K, Metabolic regulation of the adenine nucleotide pool I Studies on the transient exhaustion of the adenine nucleotides by glucose in Ehrlich ascites tumor cells *Biochim biophys Acta* (Amst) 1965 *104* 330—347

OZAWA K K SETA, H ARAKI and H HANDA The effect of ischemia on mitochondrial metabolism *J Biochem* (Tokyo) 1967 *61* 512—514

PACKER L and M G MUSTAFA Pathways of electron flow established by tetramethylphenylenediamine in mitochondria and ascites tumor cells *Biochim biophys Acta* (Amst) 1966 *113* 1—12

RACKER E Carbohydrate metabolism in ascites tumor cells *Ann N Y Acad Sci* 1956 *63* 1017—1021

REDFARN, E R and J M DIXON Spectrophotometric methods for the determination of succinic-dehydrogenase and succinic-oxidase activities in mitochondrial preparations *Biochem J* 1961 *81* 19p—20p

ROCHMAN H G H LATHE and M J LEVELL The effect of temperature and anoxia of kidney on the subsequent oxidative phosphorylation of mitochondria *Biochem J* 1967 *10?* 48—52

SCHATZ G and E RACKER Stable phosphorylating submitochondrial particles from bakers yeast *Biochem biophys Res Commun* 1966 *22* 579—584

SLATER, E C The action of inhibitors on the system of enzymes which catalyse the aerobic oxidation of succinate *Biochem J* 1949 *45* 8—13

SLATER E C Our present knowledge of the mechanism of energy conservation in biological oxidations *Bull Soc Chim biol* (Paris) 1966 *48* 1151—1168

WOJTCZAK L and A L LEHNINGER Formation and disappearance of an endogenous uncoupling factor during swelling and contraction of mitochondria *Biochim biophys Acta* (Amst) 1961 *51* 442—456

WU R and E RACKER Regulatory mechanisms in carbohydrate metabolism III Limiting factors in glycolysis of ascites tumor cells *J biol Chem* 1959 *234* 1029—1035

Acta physiol scand 1969 76 485—494

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The Regulation of Extra- and Intracellular Acid-Base Parameters in the Rat Brain during Hyper- and Hypocapnia

By

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Received 13 November 1968

Abstract

Kjallquist, Å , M Nardini and B K Siesjo *The regulation of extra and intracellular acid base parameters in the rat brain during hyper and hypocapnia* Acta physiol scand 1969 76 485—494

The influence of hyper and hypocapnia upon extra and intracellular acid base parameters in the

continuously increasing lactate concentration and since the constant buffer capacity at CO_2 tensions exceeding 50 mm Hg was associated with lactate concentrations which hardly varied at all

Recently Ponten (1966) determined the relation between the CO_2 tension and the bicarbonate concentrations of brain tissue and of cisternal cerebrospinal fluid (CSF) in phenobarbital anaesthetized rats (cf Roos 1965) The CO_2 absorption curve thus obtained for the tissue phase gave a calculated CO_2 buffer capacity, defined as d log P_{CO_2}/d pH of about 1 4 This value which is slightly lower than that of whole blood *in vitro* suggested that the tissue contains a minimum concentration of non bicarbonate buffers of about 35 mmoles kg and a buffer base concentration of about 35 meq kg of tissue water

[1] On leave of absence from the Department of Neurology and Neurosurgery University of C Italy

In the phenobarbital series referred to, control experiments indicated that the tissue lactate concentration was only slightly influenced by variations in the CO_2 tension, and the calculations of an equivalent buffer system were made on the assumption of a constant buffer base concentration (Ponten 1966) However, later experiments have shown that there are appreciable changes in the tissue and CSF lactate concentrations, especially in marked hyperventilation (Leusen and Demeester 1966, *Plum and Posner 1967, Granholm and Siesjö 1968*) The present experiments were devised to study the influence of metabolic events on the regulation of extra and intracellular acid base parameters in hyper- and hypocapnia To that end, the bicarbonate, lactate, and pyruvate concentrations of rat brain tissue and of cisternal CSF were measured In order to minimize depression of the tissue metabolism due to the anaesthesia nitrous oxide was used in all experiments Finally since changes in the phosphocreatine and ATP levels may influence intracellular acid base events (*Siesjö et al 1968*) *tissue concentrations of phosphocreatine and adenine* nucleotides were determined

Methods

Male rats of the Wistar strain weighing 300 [illegible]00 grams, were anaesthetized with divinyl ether quickly tracheotomized and maintained on [illegible]0 % nitrous oxide and 30 % oxygen Artificial ventilation was given with a Palmer Miniature Respirator Muscle relaxation was achieved with tubocurarine chloride The rectal temperature was measured with a mercury thermometer One femoral artery was cannulated for blood pressure recording with an electromanometer (ELEMA Stockholm and for blood sampling The atlanto-occipital membrane was exposed for CSF sampling and a plastic funnel was fitted into a skin incision over the cranial vault to allow freezing of the brain *in situ*

Arterial blood was analyzed at 37 C for pH P_{CO_2} and P_{O_2} using microelectrodes Radiometer *Copenhagen and Eschweiler und Co Kiel* and for haemoglobin using a standard colorimetric method Vitatron Holland The values measured were used for calculating actual and standard bicarbonate values from a conventional nomogram Siggaard Andersen 19[illegible]3

The CSF samples withdrawn ([illegible]0—80 µl were analyzed either for the total CO_2 content or for the lactate and pyruvate concentrations The CSF bicarbonate concentration and the CSF pH *were calculated from the total* CO_2 *content and from the mean tissue* CO_2 *tension* The latter was [illegible]

for changes in the CSF pH

The frozen tissue samples were analyzed for the total CO content Ponten and Siesjö [illegible] and for the lactate pyruvate ATP ADP AMP and phosphocreatine concentrations [illegible] et al 19[illegible] using extraction of the tissue at [illegible] C Usually the total CO_2 content and the water content were measured on one half of the brain and the other metabolites on the other half However in some animals the samples obtained for determinations of the water content were too small to obtain accurate measurements Therefore and since no systematic differences were found in the tissue water content at the CO tensions studied calculations were made using the mean water [illegible] series [illegible] 8 4

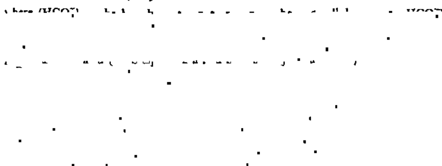
[illegible] d pyruvate between the extra and intracellular spaces [illegible] blood and extracellular fluid [illegible] d [illegible] Rall Oppelt and Patlak 19[illegible] Woodward Reed and [illegible] trations were representative of the whole extracellular [illegible] However for comparison the [illegible] bicarbonate concentrations and the equ[illegible] intracellular pH values were also calculated for extracellular volumes of 5 and 20 per cent respectively

There are theoretical objections to using the pH term in a mu[illegible] arterial [illegible] in Siesjö and Ponten 196[illegible] but in order to facilitate comparisons with results published by [illegible] an equ[illegible] al of

(apparent) intracellular pH ' (pH_i') was calculated This equivalent pH is synonymous with the pH in a one-compartment system with the same bicarbonate concentration and the same CO_2 tension as was measured in the biological system After subtracting the amount of CO_2 dissolved in the tissue the intracellular bicarbonate concentration and the equivalent intracellular pH was calculated according to the following equations

$$(HCO_3)_i = \frac{(HCO_3^-)_t - 0\ 03(HCO_3^-)_{bl} - 0\ 12(HCO_3^-)_{CSF}}{0\ 64}$$

$$pH_i' = 6\ 12 + \log \frac{(HCO_3^-)_i}{P_tCO_2\ \ 0\ 0314}$$



studied, whence all animals were treated as one group

Results

The total CO_2 content of brain tissue was measured in 35 expts while the CO_2 content of cisternal CSF was analyzed in 17 expts In all experiments the mean arterial blood pressure exceeded 110 mm Hg, the arterial haemoglobin concentration exceeded 12 5 g per 100 ml and the arterial P_{O_2} was higher than 85 mm Hg

In Fig 1 the measured CSF and tissue TCO_2 values have been related to the measured arterial CO_2 tensions, and curves have been drawn, representing the best visual fits to the experimental points The resulting curves show that at arterial CO_2 tensions of 15, 20, 30 40, 50 60, 70, 80 and 90 mm Hg, the total CO_2 content of brain tissue was 8 2, 9 6, 11 9, 13 6, 15 1, 16 5, 17 8, 19 0 and 20 1, and the total CO_2 content of cisternal CSF 19 7, 22 5, 26 9 30 1, 32 9 35 0, 36 8, 38 3 and 39 6 mMoles/kg respectively Thus for the same increase in the arterial CO_2 tension (20—90 mm Hg) the CSF CO_2 content increased by 8 mMoles/kg more than did the total tissue phase

The lines, representing the best fits to the experimental points were used for further calculations Thus, after converting the arterial CO_2 tensions to tissue CO_2 tensions and after subtracting the physically dissolved CO_2 and the CO_2 contained in the blood and in the extracellular space of the tissue, the bicarbonate concentrations of the intracellular phase were obtained

Fig 2 shows the relation between the tissue CO_2 tension and the bicarbonate concentrations of the CSF and the intracellular water phase, respectively However, the intracellular bicarbonate concentration was not only calculated for an extracellular space of 12 per cent but also for extracellular spaces of 5 and 20 per respectively Assuming that t

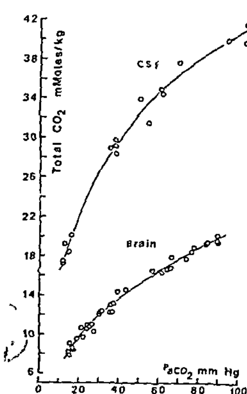


Fig 1

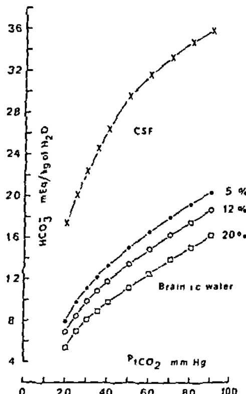


Fig 2

Fig 1 The relation between the arterial CO_2 tension and the total CO_2 content of cisternal CSF and of whole brain tissue in rats anaesthetized with nitrous oxide CSF was sampled prior to the freezing of the tissue *in situ* only in about half of the experiments The curves through the experimental points were drawn by best visual fit

Fig 2 The relation between the tissue (and CSF) CO_2 tension and the CSF and intracellular bicarbonate concentrations The latter were derived from the lines of best fit in Fig 1 by subtracting the amount of CO_2 dissolved, the amount of bicarbonate contained in the blood of the tissue (assumed to be 3 % of the weight) and the amount of bicarbonate contained in the extracellular volume The calculations were made for extracellular volumes of 5 12 and 20 % respectively

vivo conditions (see Discussion) tissue CO_2 tensions of 20, 30, 40 50 60 70 80 and 90 mm Hg corresponded to intracellular bicarbonate concentrations of 6 9 9 9, 11 8, 13 5, 15 0, 16 3, 17 6 and 18 8 meq kg of intracellular water, respectively At the same CO_2 tension the CSF bicarbonate was 17 5, 22 5, 26 5, 29 6 31 7 33 4 34 9 and 36 0 meq/kg of CSF, respectively This implies that the extracellular bicarbonate changed by 18 5 and the intracellular by 11 9 meq kg water when the tissue CO_2 tensions varied between 20 and 90 mm Hg The figure also shows that a 20 per cent extracellular space would imply both that the intracellular bicarbonate should decrease almost to 5 meq kg at a tissue CO_2 tension of 20 mm Hg and that the 70 mm Hg increase *in the CO_2 tension (see above)* should be followed by a 10 9 meq/kg increase in the intracellular bicarbonate

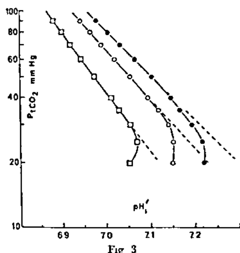


Fig 3

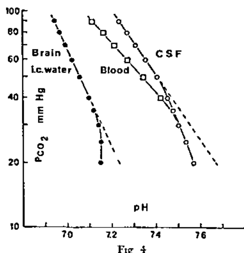


Fig 4

Fig 3 The relation between the tissue (and CSF) CO_2 tension (logarithmic scale) and the

in fig 2

In Fig 3, the calculated equivalent intracellular pH has been plotted against log P_{CO_2}. The figure shows that the buffer capacity of the intracellular phase, defined as d log P_{CO_2}/d pH, is not constant but increases continuously at low CO_2 tensions In fact, the plots show that the pH approaches constancy in hyperventilation in that a further decrease in P_{CO_2} leads to very small, if any, pH changes In hypercapnia (P_{CO_2} values higher than 40—50 mm Hg) the buffer line cannot be distinguished from a straight line, implying a constant buffer capacity In this region, assuming an ECF volume of 12 per cent, the buffer capacity can be calculated to 2 27, while ECF volumes of 5 and 20 per cent give intracellular buffer capacities of 2 04 and 2 74, respectively

In the next figure (Fig 4) the buffer lines of the CSF and the intracellular spaces have been plotted using the mean tissue CO_2 tensions and compared to the corresponding plasma P_{CO_2}/pH changes, using the arterial CO_2 tensions for the latter plot The figure shows that the buffer capacity of the intracellular space (β = 2·27) is higher than that of the CSF (β = 1 59) but very much higher than that

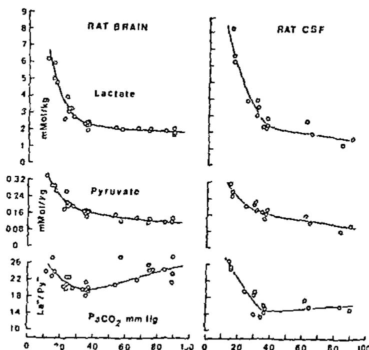


Fig 5 The relation between the arterial CO_2 tension and the lactate and puruvate concentrations as well as the lactate/pyruvate ratios of whole brain tissue and of cisternal CSF Note marked increase in the lactate (and pyruvate) concentrations at CO_2 tensions below about 30 mm Hg

rial blood ($\beta = 1\ 3$) At low CO_2 tensions, however, there were similar increases in the buffer capacity of all three systems

The relations between the arterial CO_2 tension and the lactate and pyruvate concentrations of brain tissue and cisternal CSF are given in Fig 5 and in Fig 6 the calculated intracellular lactate and pyruvate concentrations have been compared to the corresponding CSF parameters, assuming a 12 per cent extracellular volume The results confirm the lactate and pyruvate changes in hyper- and hypocapnia previously reported for cat cerebral cortex (Granholm and Siesjö 1968) but the present rat material anaesthetized with nitrous oxide, showed a larger increase in the lactate and pyruvate concentrations at low CO_2 tensions Thus the present results indicate that at arterial CO_2 tensions below 15 mm Hg the tissue lactate concentration increased to values higher than 5 meq/kg of intracellular water and the CSF lactate to values exceeding 7 meq/kg It was also indicated that there were very small further decreases in the tissue and CSF lactate and pyruvate concentrations when the CO_2 tension increased beyond 50 mm Hg

There were no significant variations in the tissue concentrations of phosphocreatine, ATP, ADP and AMP at the various CO_2 tensions studied except for a suggested increase in AMP at extreme hyperventilation These results thus confirm the results previously reported for cat cerebral cortex (Granholm and Siesjö 1969)

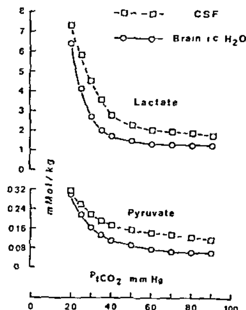


Fig 6 Comparison between the lactate and pyruvate concentrations of cisternal CSF and of the intracellular space of brain tissue The lactate and pyruvate concentrations of the intracellular space were calculated on the assumption of a 15 % extracellular volume (blood plus CSF and ECF spaces)

Discussion

The present results have shown that both the intracellular phase of brain tissue, and the CSF, are very well buffered against changes in the CO_2 tension in that the apparent buffer capacities calculated greatly exceeded that of whole blood *in vivo* The low buffer capacity of the blood *in vivo* is well known and is explained by the exhange of bicarbonate between plasma and extracellular fluids in hyper- and hypocapnia (see Schwartz and Relman 1963) The high CSF buffer capacity is explained by the fact that not only the changes in the plasma bicarbonate, but also the pH- dependent changes in the CSF/plasma potential, will favor increase in the CSF bicarbonate in hypercapnia and decrease in hypocapnia (Kjallquist and Siesjo 1967, 1968 Siesjo and Kjallquist 1969

The buffering of the tissue phases was not constant at different CO_2 tensions but increased at low CO_2 tensions to approach infinite buffering power at extreme hyperventilation The varying buffer capacity strongly indicates that pH in the extra- and intracellular spaces is regulated not only by physicochemical buffering but also by metabolic mechanisms

It appears that the metabolic control is partly exerted by variations in the lactic acid production since the increase in the calculated buffer capacity at low CO_2 tensions was parallelled by continuously increasing tisssue and CSF lactate concentrations, and since the constant buffer capacity at CO_2 tensions above 50 mm Hg was companied by [illegible]

The conclusions regarding the buffer capacity of the intracellular space, as well as regarding the change in pH or bicarbonate with the CO_2 tension, are relatively independent of any assumption with regard to the size of the extracellular volume However, the actual bicarbonate concentration, and the actual pH, calculated for the intracellular space at any given CO_2 tension are critically dependent on the size of this volume The quantitative relations discussed below are based on an extracellular volume of 12 per cent, a figure which is close to those reported by many workers, using different extracellular markers introduced in a way which circumvents the blood brain barrier and the sink action of the CSF (Rall, Patlak and Oppelt 1962, Woodward, Reed and Woodbury 1967, Davson 1967) It was also assumed that the bicarbonate, lactate and pyruvate concentrations of the cisternal CSF represented homogeneous ECF concentrations This assumption is less easily checked by control experiments but small deviations in concentrations between the CSF and the true ECF will not seriously affect the conclusions

Recently, Ponten (1966) reported an apparent buffer capacity for brains of phenobarbital-anaesthetized rats of about 1 4 while Roos (1965) calculated a buffer capacity of more than 2 0, using the DMO method for measurements on cat cerebral cortex In both these studies, the buffer capacity was equally large at all CO_2 tensions studied The main reason for the discrepancy between the present results, and those reported by Ponten (1966), may be the type of anaesthesia used, while the present finding of a buffer capacity which varied with the CO_2 tension, appears to be adequately explained by the large changes in the lactate (and pyruvate) concentrations observed during hyperventilation in the present experiments under nitrous oxide anaesthesia This is in contrast to the relatively small changes in lactate, suggested by Ponten's control measurements However, the fact that the buffer capacity, which can be calculated from the straight portion of the present log P_{CO_2}/pH curve ($\beta = 2\ 3$) greatly exceeds the buffer capacity derived from Pontén's phenobarbital anaesthetized rats ($\beta = 1\ 4$) suggests that another buffer mechanism comes into action in the nitrous oxide animals Moreover, the buffer capacity calculated in this group of animals is so high that the responsible buffers are difficult to account for in any other system than in whole blood with its high concentration of imidazole groups (see Edsall and Wyman 1958) Thus, further work is required to elucidate buffering mechanisms *in vivo*

The pK of lactic acid is so low (3 8) that the acid can be regarded as being virtually completely ionized in tissues For that reason, changes in the lactate concentrations will be accompanied by equally large changes in the buffer base concentration The same argument applies to pyruvic acid but the actual pyruvate concentrations are so low that their influence on the buffer base concentration can be neglected The lactate changes will, however by themselves lead to a variation in the buffer base concentration of 4—5 meq kg of intracellular water over the P_{CO_2} region 20—90 mm Hg The production of lactic acid at low CO_2 tensions undoubtedly lowers the bicarbonate concentration of the CSF and intracellular phases In the CSF, which lacks other buffers than the bicarbonate buffer system there will be a 1 1 relation-

ship between the appearance of lactate and the disappearance of bicarbonate Thus, if the *in vivo* buffer curve of CSF is corrected for the changes in the lactate concentration at the various CO_2 tensions the log P_{CO_2}/pH curve becomes linear with a slope, which cannot be distinguished from the upper portion of the curve displayed in Fig 4 In the intracellular space, however, the presence of nonbicarbonate buffers will give a variable relationship between the accumulation of lactate and the removal of bicarbonate The quantification of these changes will be reported in a forthcoming publication from the laboratory

The changes in the lactate (and pyruvate) concentration were the only chemical events observed, wich will lead to a change in the buffer base concentration Theoretically, such changes are possible through shifts in the steady state reactions involving the phosphocreatine creatine and the adenine nucleotide systems (Siesjo *et al* 1968), but none of the compounds involved in these reactions showed any significant variation with the CO_2 tension

The changes observed in the lactate/pyruvate ratios of brain tissue and of CSF in hyperventilation (see Fig 5) fully confirm the previous finding from the laboratory (Granholm and Siesjo 1968 a and b) that a pronounced hyperventilation leads to significant increases in the tissue and CSF lactate/pyruvate ratios These increases occur simultaneously with a greatly accelerated lactate production in a CO_2 tension range, in wich a further decrease in P_{CO_2} seems to be unaccompanied by any significant pH changes

Acknowledgements The present work was supported by the Swedish Medical Research Council (Project B68—14X—263—04) and by Carl Bertel Nathhorst s Vetenskapliga Stiftelse

Note add d in proof When the present manuscript was written up we were not aware of the fact that Weyne Demeester and Leusen (Arch internat Physiol Bioch 1968 76 415—433) had reported an increase in the tissue buffer capacity of the rat brain in hypocapnia However these authors calculated tissue pH for only three P_{CO_2} levels and no CSF measurements were performed

References

BRZEZINSKI J A KJALLQUIST and B K SIESJO Mean carbon dioxide tension in the brain after carbonic anhydrase inhibition *J Physiol (Lond)* 1966 *88* 13—23

DAVSON H *Physiology of the cerebrospinal fluid* J & A Churchill Ltd London 1967

EDSALL, J T and J WYMAN *Biophysical chemistry* Academic Press Inc New York 1958 pp 550—571

FENCL, V T B MILLER and J R PAPPENHEIMER Studies on the respiratory response to disturbances of acid base balance with deductions concerning the ionic composition of cerebral interstitial fluid *Amer J Physiol* 1966 *210* 459—472

G[illegible]

G[illegible]

GRANHOLM L and B K SIESJO The effect of hypercapnia and hypocapnia upon the cerebrospinal [illegible]

K[illegible]

LEUSEN I and G DEMEESTER Lactate and pyruvate in the brain of rats during hype[illegible]
Arch int Physiol

MITCHELL, R A, D A HERBERT and C. T CARMAN, Acid base constants and temperature coefficients for cerebrospinal fluid *J appl Physiol* 1965 *20* 27—30
PLUM, F and J B POSNER, Blood and cerebrospinal fluid lactate during hyperventilation *Amer J Physiol* 1967 *212* 864—870
PONTÉN U, Acid base changes in rat brain tissue during acute respiratory acidosis and baseosis *Acta physiol scand* 1966 *68* 152—163
PONTÉN, U and B K SIESJÖ, A method for the determination of the total carbon dioxide content of frozen tissue *Acta physiol scand* 1964 *60* 297—308
PONTÉN U and B K SIESJÖ, Gradients of CO_2 tension in the brain *Acta physiol scand* 1966 *67* 129—140
RALL, D P, W W OPPELT and C. S PATLAK Extracellular space of brain as determined by diffusion of inulin from the ventricular system *Life Sci* 1962 *2* 43—48
ROOS A, Intracellular pH and intracellular buffering power of the cat brain *Amer J Physiol* 1965 *209* 1233—1246
SCHWARTZ W B and A S RELMAN, A critique of the parameters used in the evaluation of acid base disorders *New Eng J Med* 1963 *268* 1382—1388
SIESJÖ B K, The solubility of carbon dioxide in cerebral cortical tissue of cats With a note on the solubility of carbon dioxide in water, 0 16 M NaCl and cerebrospinal fluid *Acta physiol scand* 1962 a *55* 325—341
SIESJÖ B K, The bicarbonate/carbonic acid buffer system of the cerebral cortex of cats as studied in tissue homogenates II The pK_1 of carbonic acid at 37 5° C, and the relation between carbon dioxide tension and pH *Acta neurol scand* (Kbh) 1962 a *38* 121—141
SIESJÖ, B K, J BRZEZINSKI, Å KJÄLLQUIST and U PONTÉN, Carbon dioxide and acid base equilibria in brain tissue In *Brain Edema* Ed by Klatzo, J and Seitelberger, F Springer Verlag New York Inc 1967
SIESJÖ, B K, A E KAASIK, L NILSSON and U PONTÉN, Biochemical basis of tissue acidosis *Scand J clin Lab Invest* 1968 Suppl 102 III A
B K and Å KJÄLLQUIST, A hypothesis for the regulation of extracellular pH in the brain. *J Physiol (Lond)* 1969
SIESJÖ B K and U PONTÉN, Intracellular pH—true parameter or misnomer? *N Y Acad of Sci* 1966 *133* 78—86
SIGGAARD-ANDERSEN O, Blood acid base alignment nomogram *Scand J clin Lab Invest* 1963 *15* 211—217
WOODWARD D L, D J REED and D M WOODBURY Extracellular space of rat cerebral cortex *Amer J Physiol* 1967 *212* 367 370

Acta physiol. scand. 1969. 76. 495—502
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Turnover of H^3-Labelled Palmitate in the Unanesthetized Rat

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Received 15 November 1968

Abstract

BOBERG, J *Turnover of H^3 labelled palmitate in the unanesthetized rat* Acta physiol scand 1969 *76* 495—502

The turnover rate of blood plasma FFA has been studied in unanesthetized rats by single injection as well as constant infusions of albumin bound palmitic 9 10 H^3 acid The turnover was found to be about 50 μmoles/min per kg body weight with both techniques The mean half life time of FFA was estimated to 17 sec with no significant difference between fed and fasted rats

For the quantitation of the turnover of free fatty acids (FFA) in blood plasma with isotopes two techniques are available In the first a single i v injection of labelled albumin bound fatty acid is given and the initial disappearance of radioactivity in plasma FFA is followed (Havel and Fredrickson 1956 Laurell 1957, Fredrickson and Gordon 1958 Borgstrom and Olivecrona 1961 Baker and Schotz 1967) Steady state conditions are assumed and the obtained fractional turnover of FFA is multiplied by the blood plasma pool of FFA The second technique utilizes a constant infusion of labelled albumin bound FFA and the turnover is calculated from the infusion rate and the specific activity of the plasma FFA during steady state conditions (Armstrong *et al* 1961) Both these techniques have so far been difficult to perform on unanesthetized rats

In this report the fractional turnover and the turnover rate of FFA in unanesthetized rats have been estimated using tritium labelled palmitate The rats were equipped with chronic catheters in the right jugular vein and in the abdominal aorta (Weeks 1962 Weeks and Davis 1964)

Procedures

Animal procedure Male Sprague-Dawley albino rats (Anticimex AB Sweden) weighing 150—250 g were used During anesthesia with sodium pentobarbitone (about 60 mg per kg body weight intraperitoneally one catheter was placed in the right jugular vein and a second in the abdominal aorta The catheters and the surgical procedure were similar to those of Weeks and Davis (1964) All experiments were performed when the rats had regained the weight loss caused by the surgery i e 5 10 days after operation (Fig 1)

The fed rats had free access to water and their ordinary food (pellets from Anticimex AB Sweden) The fasted rats had water ad libitum but no food during the 20—22 hrs before the experiments were started which always was about nine o clock a m During the stud es the rats were chained but could move freely (Weeks 1962) Their appearance was normal and they did not seem to be upset by the experiment

Dose procedure Palmitic 9,10 H^3 acid (New England Nuclear Corp, Mass, USA) with a specific activity of 500 mCi per mmole was used The isotope was purified by means of thin layer chromatography (TLC) (Boberg 1966) and then stored dissolved in heptane at —20° C under N_2 before use When the purified material was subjected to chromatography on TLC (Boberg 1966) only one spot was visible on the chromatogram and 99 5 per cent of the radio-

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found as methyl palmitate

In the single injection studies 50 μCi of H^3 labelled
tube, the solvent evaporated at about 60° C with a ge
molar NaOH solution added and the palmitic acid
palmitate was cooled at room temperature to 35—40°

total dose 0 15—0 30 ml was given as rapidly as possible (1—2 sec) into the jugular vein catheter without flushing The catheter volume was measured (around 0 01 ml) and the radioactivity corresponding to this volume was corrected for

In the constant infusion studies the palmitate serum complex was prepared as described above A primer dose of about 5 μCi of H^3 palmitate was given i v and within 15 sec the constant infusion was started The infusion rate was 0 03 ml/min corresponding to about 5 μCi of H^3 palmitate per min

Sampling procedure Blood was sampled from the aortic catheter (volume around 0 01 ml) Systemic blood pressure forced blood through the catheter into heparinized glass capillary tubes 75×1 mm (hematocrit tubes) In the single injection studies blood was allowed to flow freely during the first minute after the injection and capillary tubes were changed once every 5 to 6 sec Between further samples a constant infusion of 0 03 ml per minute of saline was used to keep the catheter patent The amount of blood was estimated by weighing the capillary tubes The blood samples weighed about 30 mg The blood was flushed out of the capillary tubes into test tubes with diluted human serum (blood bank serum and saline 1 1) and made up to a volume of 3 ml One ml was used for determination of the J^{12} radioactivity and 1 ml was extracted for determination of radioactivity in the FFA fraction

Before the isotope injection and at the end of the experiment aortic blood was taken for hematocrit determination At the end of the experiment the rat was killed by a blow on the head and 2—5 ml of blood was withdrawn by aortic puncture into heparinized tubes In some experiments this amount of blood was taken from the aortic catheter with the rat still alive There seemed to be no consistent difference if blood was taken by aortic puncture or directly from the catheter The blood was centrifuged and the plasma was extracted for titration of FFA

Analysis J^{125} gamma ray activity was determined in a Well scintillation counter essentially as described by Wetterfors *et al* 1962) Radioactivity in FFA was determined in a Packard liquid scintillator after extraction in chloroform methanol and separation by TLC according
to carrier lipids (0 5
mg were added to the
ex *t al* (1960)
Radioactivity of
of distribution of
Specific gravity of whole blood was taken as 1 05
matocrit value of the sample taken at the end of the
plasma volume

[1] Kindly performed by Dr K Green
[2] Kindly supplied by G Birke M D and L O Plantin chemical research engineer

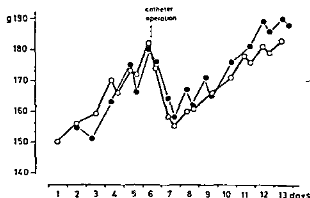


Fig 1 Body weight of 2 rats before and after the implantation of catheters

Calculation of the fractional turnover rate of palmitic acid was done from the linear regression line (calculated by least squares) of blood FFA radioactivity plotted semilogarithmically versus time The radioactivity of the samples taken between 15 and 50 sec after injection was used for this calculation Turnover rate by constant infusion technique was calculated as described by Armstrong *et al* (1961) Statistical analyses were performed according to Snedecor (1956)

Results

Animal operation technique Following the catheter operation the rats lost 10 to 15 per cent of their body weight However, after 24 hrs they began to gain weight again at almost the same rate as before operation (see Fig 1) A 150 g rat gained about 6 g/day Since rats eat preferentially during the night their weights are about 3 g higher in the morning than in the afternoon Experiments were not performed until the animals had reached their preoperative weight If this did not occur within 10 days, the rat was not used About 1 out of 4 operated rats was rejected because of not functioning catheters

Turnover of plasma FFA by single injection technique in fed rats The J^{125} radioactivity stayed on a plateau 10 to 15 sec after a single injection of the labelled palmitate and albumin (see Fig 2) and the radioactivity of the plasma FFA disappeared single exponentially for 40 to 60 sec The value estimated from the intercept of the extrapolated exponential curve in Fig 2 is 87 per cent of the given dose This is in agreement with what Fredrickson and Gordon found in man (1958) and is explained by the fact that loss of radioactivity occurs before mixing in the plasma is completed The intercept value should be still lower if one consider the 2 to 4 sec the blood flow through the aortic catheter delays the sampling The results from 11 fed rats are seen in Table I The blood volume of these rats was 13.8 ± 0.6 ml and the plasma volume 7.8 ± 0.5 ml 6.9 and 3.9 ml per 100 g b w respectively These values are in agreement with those earlier reported (Cartland and Koch 1928)

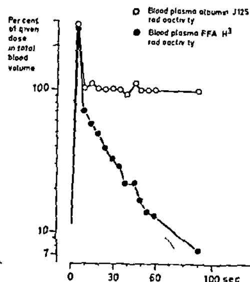


Fig 2 Blood plasma radioactivity of albumin J^{125} and FFA H^3 after a single injection of J^{125} labelled human albumin and H^3 labelled serum bound palmitate to a rat The extrapolated curve is the linear regression line calculated (by least squares) from the samples taken between 15 and 50 sec after the injection

TABLE I Data from the experiments on the fed not anesthetized rats Analysis and calculations were performed as is described under Procedures

Rat no	Body weight g	Hematocrit %	Blood volume ml	Plasma volume ml	FFA concentration µmoles/ml	FFA plasma pool µmoles	FFA fractional turnover rate min^{-1}	FFA turnover rate µmoles/min	FFA turnover rate µmoles min and kg b w
1	171	41	13 3	7 8	0 27	2 11	—		
2	182	40	14 9	8 9	0 42	3 75	2 38	8 94	49 1
3	190	45			0 31	—			—
4	184	56	14 1	6 2		—	1 96		
5	208	40	17 0	10 2	0 39	3 98	2 97	11 82	56 8
6	173	53	11 1	5 2			1 99	—	
7	213	42	11 6	6 7	0 47	3 16	2 54	8 03	37 7
8	220	42	14 1	8 2	0 47	3 84	2 84	10 92	49 6
9	207	39	13 0	7 9	0 52	4 12	2 03	8 37	40 4
10	211	42	12 0	7 5	0 58	4 34	2 65	11 50	54 5
11	243	42	15 5	9 0	0 44	3 96	2 89	11 43	47 0
Mean	200	44	13 8	7 8	0 43	3 66	2 47	10 14	47 9
Sem	7	2	0 6	0 5	0 03	0 25	0 13	0 61	2 6

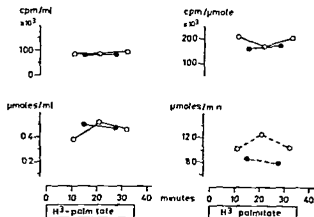


Fig 3 Radioactivity, concentration, specific radioactivity and turnover rate of blood plasma FFA in 2 rats during a constant infusion of H^3 labelled palmitate At zero time a primer dose of about 5 μCi was given and within 15 sec the constant infusion was started Infusion rate was 0.03 ml/min (about 5 μCi)

The concentration of plasma FFA in the rats was 0.43 ± 0.03 μmoles/ml and the plasma pool of FFA was 3.66 ± 0.25 μmoles These values are high, *e g* in comparison to values seen in carbohydrate fed rats This may be explained by the eating habits of the rats, which will be commented upon in the discussion The fractional turnover rate was 2.47 ± 0.13 min^{-1} corresponding to a half life of about 17 sec The resulting total turnover rate was *10.14 ± 0.61* μmoles/min which is *47.9 ± 2.6* μmoles/min and kg body weight

Turnover rate of plasma FFA by constant infusion technique in fed rats The results from 2 rats are seen in Fig 3 During the time studied the radioactivity of plasma FFA (cpm/ml) was constant. The concentration of plasma FFA in these rats ranged between 0.38 and 0.52 μmoles/ml The means of the turnover rates were of the same order of magnitude as those determined after a single injection One rat had a mean turnover rate of 11.1 μmoles/min and the other 8.3 μmoles/min which is 45.0 and 40.7 μmoles/min and kg b w respectively

Turnover rate of plasma FFA by single injection technique in fasting rats The picture of the elimination curves was the same as for the fed rats The results from 10 fasting rats are seen in Table II The blood volume was 12.3 ± 0.4 ml and plasma volume 6.8 ± 0.3 ml which is 6.8 and 3.8 ml per 100 g b w respectively—almost the same as for the fed rats The concentration of plasma FFA was 0.49 ± 0.06 μmoles/ml which is lower than has been described from other laboratories for rats fasted 20 to 22 hrs (McCalla *et al* 1957 Laurell 1959 a b Borgstrom and Olivecrona 1961 Barrett 1964) However this value on arterial plasma FFA concentration agrees well with what has been found in other studies reported from our laboratory (Carlson and Nye 1966) The FFA plasma pool had a value of 3.36 ± 0.36 μmoles The mean fractional turnover rate was 2.45 ± 0.14 min^{-1}—very similar to the mean value of the fed rats The total turnover rate was 8.69 ± 1.61 μmoles min or 47.4 ± 8.9 μmol min and kg body weight This value is very similar to the turnover for the fed but there is a wider range

TABLE II Data from the experiments of the fasted not anesthetized rats Analysis and calculations were performed as is described under Procedures

Rat no	Body weight g	Hematocrit %	Blood volume ml	Plasma volume ml	FFA concentration µmoles/ml	FFA plasma pool µmoles	FFA fractional turnover rate min⁻¹	FFA turnover rate µmoles/min	FFA turnover rate µmoles/min and kg b w
23	181	—	13.4	—	—	—	2.12	—	—
24	167	44	12.3	6.9	—	—	3.02	—	
25	176	50	12.0	6.0	0.56	3.36	1.93	6.48	36.8
26	182	45	12.7	7.0	0.61	4.27	2.15	9.18	50.4
27	162	47	9.8	5.2	—	—	2.35	—	—
28	178	43	12.4	7.1	0.39	2.77	2.24	6.20	34.8
29	183	41	11.9	7.0	0.65	4.55	3.25	14.79	80.8
30	177	44	12.6	7.1	0.35	2.49	—	—	—
31	199	42	13.3	7.7	0.35	2.70	2.51	6.78	34.1
32	194	40	—	—	—	—	2.50	—	—
Mean	180	44	12.3	6.8	0.49	3.36	2.45	8.69	47.4
sem	3	1	0.4	0.3	0.06	0.36	0.14	1.61	8.9

Discussion

The plasma volumes of the rats studied had values very similar to what has been described before

The concentration of plasma FFA in the fasted rats were of the same order of magnitude as have been found in other series of rats studied at our laboratory (Carlson and Nye 1966) In the fed rats however plasma FFA concentration was higher than expected This might be explained by the fact that rats generally eat during the night i e these free fed rats had finished eating 3—6 hrs before the experiment According to the results of Barrett (1964) the plasma FFA concentration in the rat had increased twofold already after 6 hrs fasting

The removal rate of FFA from the blood stream in the rat is rapid compared to other species In this study the mean half life was 17 sec (Laurell 1959 b) gave an upper limit of 28 sec for glucose fed rats and 54 sec for rats starved for 48 hrs Borgström and Olivecrona (1961) gave a value of 48 sec for glucose fed rats However in those studies the samples were taken with about 1 min s interval after a single injection of labelled FFA and radioactivity was measured in the total plasma lipids As seen from this study the disappearance of injected labelled palmitate did not follow a 'single exponential' function longer than for 1 min after injection This was pointed out by Laurell (1959 b) and he claimed from his results that the exponential course must have occurred already at 2 1 min after the injection In the studies done by Baker and Schotz (1967) they took their first sample 1 min after the injection and

found in this sample 30 ± 4 8 per cent of injected C^{14}-palmitate, which is about twice the amount found in this study

The rapid removal rate found in the single injection studies was confirmed by the results from the constant infusion studies, which gave a similar value for the turn over

The turnover rate of plasma FFA in the fasting rats were 47 4 μmoles/min and kg b w If the FFA underwent complete oxidation the oxygen consumption should be 47 4 60 · 23 · 32 · 10^{-6} = 2 1 g O_2/hr and kg b w This value agrees well with the figures for oxygen consumption of fasting rats weighing between 120 and 201 g, which has been described to range from 1 5 to 2 1 g/hr and kg b w (Carr and Krantz 1949)

Most earlier studies on FFA turnover in the rat have been performed on anesthetized rats and venous blood samples have been taken The rats in this study had chronic catheters and underwent a surgical procedure about one week before the experiments were performed and thus cannot be considered as normal rats However, this technique seems suitable for in vivo tests in studying *e g* drugs and conditions which affect FFA turnover in the rat

Supported by grants from Reservationsanslagen Karolinska Institutet Nationalföreningen mot Hjärt och Lungsjukdomar and the Swedish Medical Research Council (19X 204 04, B69 19P 2628 01)

References

ARMSTRONG D T R STEELE N ALTZULER A DUNN J S BISHOP and R C DE BODO

plasma of rats *Brit J Pharmacol* 1964 *22* 577—584

BOBERG J Separation of labelled plasma and tissue lipids by thin layer chromatography A quantitative methodological study *Clin chim Acta* 1966 *14* 325—344

BORGSTROM B and T OLIVECRONA The metabolism of palmitic acid 1 C^{14} in functionally hepatectomized rats *J Lipid Res* 1961 *2* 263—267

CARLSON L A and E R NYE Acute effects of nicotinic acid in the rat 1 Plasma and liver lipids and blood glucose *Acta med scand* 1966 *179* 453—461

CARR J C and J C KRANTZ JR *Metabolism* In The rat in laboratory investigation 1949 (Ed E J Farris and J O Griffith Jr) J B Lippincott Company Philadelphia

CARTLAND G F and C KOCH A micro-modification of the Keith Rowntree plasma dye method for the estimation of blood volume in the rat *Amer J Physiol* 1928 *85* 540—545

FREDRICKSON D S and R S GORDON The metabolism of albumin bound C^{14} labelled unesterified fatty acids in normal human subjects *J clin Invest* 1958 *37* 1504—1515

HAVEL R J and D S FREDRICKSON The metabolism of chylomicra 1 The removal of palmitic acid 1 C^{14} labeled chylomicra from dog plasma *J clin Invest* 1956 *35* 1025—1032

LAURELL S Turnover rate of unesterified fatty acids in human plasma *Acta physiol scand* 1957 *41* 158—167

LAURELL S Distribution of C^{14} in rats after intravenous injection of non-esterified palmitic acid 1 C^{14} *Acta physiol scand* 1959 a *46* 97—106

LAURELL S Recycling of intravenously injected palmitic acid 1 C^{14} as esterified fatty acid in the plasma of rats and turnover rate of plasma triglycerides *Acta physiol scand* 1959 b *47* 218—232

McCALLA C H S GATES JR and R S GORDON JR $C^{14}O_2$ excretion after the administration of albumin bound palmitate 1 C^{14} to intact rats *Arch B*

346—357

McFarlane, A. S., Efficient trace labelling of proteins with iodine. *Nature* (Lond.) 1958. *182*. 53.
Sherrington, C. S. and S. M. Copeman, Variations experimentally produced in the specific gravity of the blood. *J. Physiol.* (Lond.) 1893. *14*. 52—96.
Snedecor, G. W., *Statistical methods*. The Iowa State University Press, Ames. Iowa. 1956.
Trout, D. L., E. H. Estes Jr. and S. J. Friedberg, Titration of free fatty acids of plasma. A study of current methods and a new modification. *J. Lipid Res.* 1960. *1*. 199—202.
Weeks, J. R., Experimental morphine addiction. Method for automatic intravenous injections in unrestrained rats. *Science* 1962. *138*. 143—144.
Weeks, J. R. and J. D. Davis, Chronic intravenous cannulas for rats. *J. appl. Physiol.* 1964. *19*. 540—541.
Wetterfors, J., S. O. Liljedahl, L. O. Plantin and G. Birke, Catabolism and distribution of albumin in gastric cancer. *Acta med. scand.* 1962. *172*. 163—186.

Acta physiol. scand. 1969. 76. 503—512
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Unit Responses in the Cochlear Nucleus of the Rat to Sweep Tones

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Received 19 November 1968

Abstract

MOLLER A R *Unit responses in the cochlear nucleus of the rat to sweep tones* Acta physiol scand 1969 *76* 503—512

Unit discharges in the cochlear nucleus of the rat have been studied in response to stimulation with continuous tones whose frequency was changed linearly at different rates (sweep tones) At low sweep rates the distribution of discharges as a function of tone frequency was an expression of the unit s response area When the rate of frequency change was increased the response areas of the unit became narrower At a certain sweep rate the probability of firing within a narrow frequency range around the units CF reached a maximum value which was several times greater than that found for low sweep speed The direction of the change in tone frequency had no significant influence on the results and the average firing rate appeared to be independent of the sweep rate These results indicate that spectral resolution of cochlear nucleus units was enhanced at a certain rate of frequency change Additional experiments showed that the enhancement of spectral resolution at a certain rate of frequency change remains upon introduction of masking wide band noise

The natural stimulation to which the auditory system is exposed and analyzes is not of constant amplitude or spectrum Since in fact the spectra of most natural sounds varies rapidly it is important to know how the auditory system processes such signals

The sounds which flying bats produce and use in their elegant echolocation system are special examples of sounds whose spectra vary rapidly Griffin (*cf* 1958) has described these sounds and their functional use in avoiding obstacles and in catching insects Bat sounds are usually pure tones emitted in short bursts and with a frequency that varies over more than one octave during a few milliseconds

A number of theories on how these sounds are perceived by the bat have been developed (Strother 1961 van Bergeijk 1964 and Nordmark 1961) but the processing by the auditory system of tones whose frequency varies rapidly has however, only recently been studied by electrophysiological methods (Suga 1965 a b c)

Current knowledge leads us to think of the auditory system as a sound analyser that is based on the frequency selectivity principles of the

brane in the cochlea. The resonant action of each point of the basilar membrane is usually believed to be analogous to that of a set of filters consisting of linear elements (see e.g. Flanagen 1960). Although there has been considerable doubt as to the bandwidth of these filters it is clear that such filters do not satisfactorily transmit signals of rapidly varying frequency (see e.g. Kupfmuller 1949). This inability reflects on the relationship that the more narrow the filter and the more rapid the frequency changes the more the response will be spread out in time.

The aim of the present investigation was to investigate how the auditory system at the level of the cochlear nucleus processes tones whose frequency varies rapidly and to determine whether the speed and direction of frequency variation influences the responses. Recordings were made from the cochlear nucleus in the white rat to continuous tones whose frequency varied linearly (linear sweep tones) as a function of time. The speed of variation encompassed a large range.

Methods

White rats weighing about 250 g were used. The surgical procedure and methods for acousti ... Møller 1969 a) and there ... iperitonelly injected ... removed by suction ... rat was placed in ... one inch condenser microphone (type 4131) together with a probe microphone (B & K type 4134, see Møller 1969 a). The one inch microphone served as a sound source. The animal was placed in a sound and vibration insulated box.

Glass microelectrodes drawn to tip diameters ranging from 1 to 5 μ and filled with 2.7 M KCL were used for recording. The electrode was positioned under visual control and it was then advanced through the cochlear nucleus in a nearly dorso-ventral direction by a hydraulic system which was operated from outside of the sound insulated box.

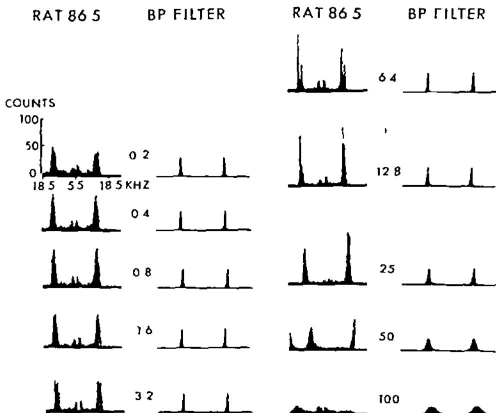
When contact with a unit was established its response area (tuning curve) was determined using 50 msec tone bursts (the intensity of which was set in 5 dB steps and whose frequency was varied in order to determine the unit's threshold). The tonebursts were presented at a rate of 6 per second. The stimulus response relationship was determined by presenting 32 tone bursts (50 msec long) at 5 dB intervals. Action potentials were recorded on magnetic tape for later processing.

Sweep tones were produced by a Wavetek (type 104) Generator in which the frequency was varied by applying a triangular wave to its frequency control input. The frequency of the triangular signal was usually varied stepwise from 0.1 to 100 Hz and sweep tones were presented for 1 minute durations.

Recorded data was processed by a TMC (type 400 A) Computer of Average Transients used in its post stimulus time histogram mode. The analyzer was triggered by a pulse which coincided with the beginning of the falling frequency sweep of the tone stimulation. Analysis time on the analyzer was set in accordance with the frequency of the sweep signal and in such a way that the sweep on the analyzer always covered the same tone frequency range. The pulse which triggered the analyzer reset the analyzer's address register immediately before it triggered a sweep. This arrangement made it possible to use only a fraction of the analyzer's 400 addresses. In that way an optional smoothing of the data could be obtained.

Results

The present study is based on recordings from more than 200 units. The units were all encountered in the dorsal and ventral areas of the cochlear nucleus of the rat. Thirty units in 8 rats were selected for detailed analysis with sweep tones in a large range of sweep rates. The units had their characteristic frequency (CF) distributed in the range from 1500 Hz to 30 kHz which is near the upper frequency limit of



response Among these units 26 had a tuning curve of the normal type *i e* asymmetrical with a steep high frequency skirt and a much less steep low frequency part and four had broad and symmetrical tuning curves All units gave a sustained respon e to tonebursts (*cf* Moller 1969 a) Responses to sweep tones from a typical experiment are shown in the left column of Fig 1 The frequency of the tone was varied linearly from 5 5 kHz to 18 5 kHz and the characteristic frequency of the unit was located in the middle of that range The sweep rate was set between 0 2 and 100 Hz as indicated by legend numbers in Fig 1 Thus the speed of frequency variation ranged from 5 2 kHz/sec to 2600 kHz/sec Each channel of the analyzer covered a frequency range of 208 Hz At low sweep rates the shape of the histograms was independent of the sweep rate as seen in Fig 1 Therefore at these sweep r the histogram represented the response area of the unit or rather its iso-r

tions (cf Sachs and Kiang 1968) Fig 1 shows that when the speed of frequency change was increased above a certain value, the shape of the histograms changed The peaks of the histograms grew in height and reached maximum values before falling again when the sweep rate was further increased It was also seen that the width of the peaks in the histograms becomes narrower as the height of the peak increases

The function of the periphery of the auditory system has often been compared to that of a resonant filter (electrical or mechanical) made up of linear elements Such an analogy may be valid for the mechanical events of the basilar membrane but it is rather doubtful whether this concept is also valid for unit responses in the eight nerve and the cochlear nucleus whose characteristics not only depend on the mechanical events of the basilar membrane but are also influenced by inhibition (Nomoto *et al* 1964, Greenwood and Maruyama 1965 Sachs and Kiang 1968)

When a linear filter is excited by sweep tones of the same type as the stimuli used in these experiments the amplitude of the output decreases when the sweep rate is increased above a certain limit This relationship is depicted in Fig 1 where the second and fourth columns show the response of a bandpass filter to sweep tones of the same characteristics as used in the *electrophysiological experiments The center frequency of the bandpass filter was equal to the* CF of the unit whose response is shown in Fig 1 Bandwidth of the filter was 750 Hz which is also in the same range of magnitude as the width of the unit s response area (Møller 1969 b) (The filter was a three pole minimum overshoot filter, Voss 1954) The properties of unit responses to stimulation with sweep tones thus differed radically from that of a linear filter

The properties of the unit responses is further illustrated in Fig 2 A which shows the height of the peaks of the histograms shown in Fig 1 (filled circles) The amplitude scale is a relative one and the response to tones of low sweep rate is set at 1 0 The dashed curve represents the unit s response obtained when the tone frequency was falling and the solid curve represents its response to a rising frequency For comparison height of the response of the bandpass filters is also shown (open circles) Fig 2 B shows the average discharge rate in spikes per second

It is seen that the peak of the histogram (Fig 2 A) reaches a height which is nearly three times greater than that obtained at low sweep rates while the average firing rate (Fig 2 B) is nearly constant throughout the ranges of sweep rates investigated This response pattern is typical and was found in all the units investigated with tuning curves of the normal type *i e* asymmetrical with a steep high frequency skirt and a slowly falling low frequency part (see Møller 1969 a)

Even units with a CF near the upper hearing limit (about 30 kHz in rats see Møller 1969 a) showed the same pattern of peak height increase in the histograms Histograms of a unit with a CF of 29 3 kHz and a normal narrow tuning curve are shown in Fig 3 The sound intensity at the units CF was approximately 20 dB above the unit s threshold

In some experiments a broad band masking noise was introduced in order to find *out how such* an additional stimulation would influence the response to sweep tones The results from a typical experiment is shown in Fig 4 and 5 The left column in Fig 4 shows the histograms of the response to sweep tones of various sweep rate (indicated by legend numbers) The upper left histogram represents 2 min of response while all the other are based on a recording time of 1 min The two columns to the right show the responses to sweep tones with the same charac

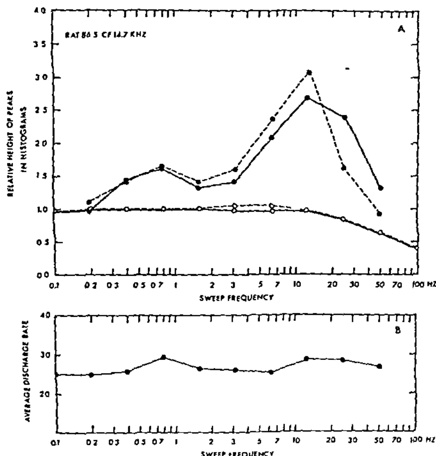


Fig 2 A Filled circles show the relative amplitude of the peaks in the histograms of Fig 1 as a function of sweep rate Solid line represents increasing frequency sweep and dashed line a decreasing sweep Open circles indicate the peak amplitude of the response of an electrical filter the responses of which are depicted in Fig 1
B Average firing rate (in discharges per second as a function of sweep frequency of the same unit

teristics but with a masking noise superimposed The signal to noise ratio was -6 and -16 dB respectively It should be mentioned that the noise and tones were passed through the same sound generating system which had a non uniform frequency response and thus colored both noise and tones in the same way The signal to noise ratio in a 1 Hz broad band was 30 dB and 20 dB respectively

As these figures indicate the histograms obtained during stimulation with masked sweep tones have nearly the same shape as those without masking but the peaks were lower and activity is also present between the peaks (Notice the difference in the vertical scale of the left column and the two columns to the right in Fig 4) Fig 5 A shows in the same way as Fig 2 A the relative height of the two peaks

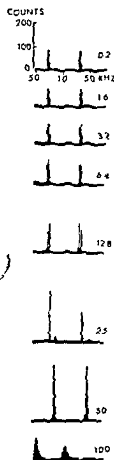


Fig 3

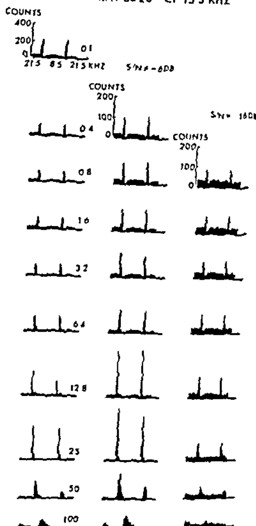


Fig 4

Fig 3 Similar records as in Fig 1 for a unit with a CF of 29 3 kHz Sound intensity at CF was 20 dB above the unit's threshold

Fig 4 Histograms of the responses of a unit to sweep tones showing the effect of masking with broad band noise Left column no masking middle and right columns signal to noise ratio of -6 and -16 dB respectively which corresponds to a signal to noise ratio in a 1 Hz band at the units CF of 30 and 20 dB respectively The tone had an intensity of 33 dB SPL at the units CF Recording time 1 minute except upper left histogram where it was 2 min Legend numbers indicate sweep frequency

the histograms Without masking the peaks reached a maximal value which was four times higher than that at low sweep tones When noise was superimposed the maximal value was lower than that without noise but there remained a pronounced peak at a signal to noise ratio of -16 dB Also in this unit the average firing rate was insignificantly influenced by the sweep rate as can be seen from Fig 5 B

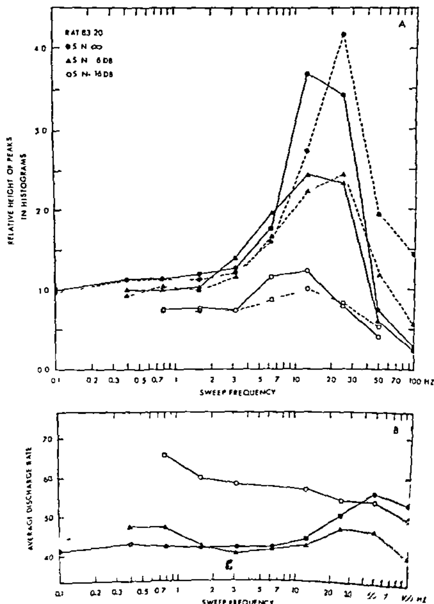
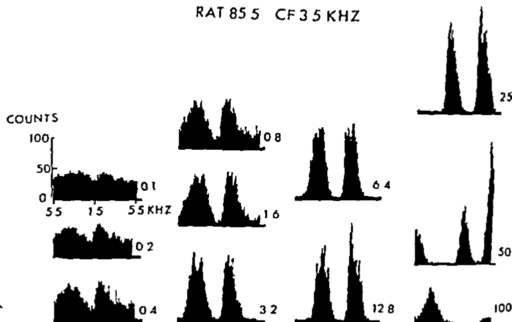


Fig 5 A The relative amplitude of the peaks in the histograms in Fig 4 drawn in the same way as in Fig 2 A
B Average firing rate (in discharges per second) as a function of sweep frequency

The results presented thus far are typical for units of the so-called common ... (see Møller 1969 a) which have an asymmetrical tuning curve with a steep ... frequency skirt and a much less steep low frequency part. A few units ... show much less frequency selectivity and so it was often difficult to de...



6 Histograms of the responses to sweep tones of a unit which had a very broad response area and a high spontaneous activity *The sound intensity was 47 dB SPL at 3 5 kHz*

response areas by listening to the discharges through a loudspeaker or watching the discharge pattern on an oscilloscope while tone bursts were presented When stimulated with sweep tones of low sweep rate such units do not show any marked frequency selectivity Conversely when the sweep rate was increased a marked selectivity can often be seen as illustrated in Fig 6 This figure displays histograms from a unit which had a diffuse response area with a broad maximum around 3 5 kHz. Such an *increase in frequency selectivity with increasing sweep rate* can be seen in the range of sweep rate from 0 1 to 50 Hz At 50 Hz the histogram becomes asymmetrical and an upgoing frequency sweep evokes a much higher and narrower peak than a downgoing sweep At a sweep frequency of 100 Hz only one peak remains (Note that there is a shift of the peaks to the right with increasing sweep rate, so that when 50 Hz is reached some of the discharges evoked by increasing tone frequency appear in the beginning of the histogram At the 100 Hz sweep frequency this shift is so big that the whole peak evoked by increasing tone frequency occupies the left part of the histogram) Also in this unit the average firing rate was almost constant and independent of the sweep rate

Discussion

The present study shows that there is a rate of frequency change at which the probability of unit discharges is higher within a narrow range around the units CF than at any other sweep rate The average discharge frequency appears to be independent of sweep rate This finding indicates that the spectral resolution of a

unit is enhanced at a certain rate of frequency change Thus the response pattern of a cochlear nucleus unit to sweep tones cannot be regarded as a result of the filter action of the basilar membrane if only that membrane resembled a filter made up of linear elements In some neurons in the cochlear nucleus and the inferior colliculus of the bat the threshold for frequency modulated tonebursts differs from that of tonebursts of constant frequency (Suga 1965 c) This difference was assumed to be due to interactions between the unit's inhibitory and excitatory response areas Further, it has been shown that the excitatory spatial response areas of the units in the eight nerve and the cochlear nucleus is 'sandwiched' between inhibitory areas (see *e g* Sachs and Kiang 1968) It is likely that the behaviour of the unit responses described in the present investigation is due to the temporal characteristics of the unit s inhibitory and excitatory response areas The organisation of the response areas of units in the auditory periphery thus show similarities to those of the receptive fields in the visual cortex as shown by Hubel and Wiesel (1959) There is a further analogy between the response patterns to sweep tones of auditory units in the cochlear nucleus and those of visual cortical units which respond to movements (Hubel and Wiesel 1965, 1968)

Since the results of the present study shows that the responses of the cochlear nucleus units is only, to small extent influenced by the direction of the sweep it is not likely that the sharpening of the response areas was accomplished by a mechanism similar to that of a collapsing filter used in certain types of radar (FM radar) as has been proposed by Strother (1961) van Bergeijk (1964) and McCue (1966)

An increase in the height of the histogram peaks was noted at the same sweep rates whether or not wide band noise was superimposed on the sweep tones It should be noted however, that this increase in height was less pronounced at very low signal to-noise ratios

The present study was supported by grants from the Swedish Medical Research Council (Grant B68-14X 90-03) Therese and Johan Andersons Stiftelse and from Magnus Bergvalls Stiftelse The Computer of Average Transients (TMC type 400 A) has been generously placed at my disposal by Professor Lars Leksell Department of Neurosurgery Karolinska Sjukhuset

References

VAN BERGEIJK W A Sonic pulse compression in bats and people A comment *J acoust Soc Amer* 1964 *36* 594—597

FLANAGAN J L Models for approximating basilar membrane displacement *Bell Syst Tech J* 1960 *39* 1163—1192

GREENWOOD D D and N MARUYAMA Excitatory and inhibitory response areas of auditory neurons in the cochlear nucleus *J Neurophysiol* 1965 *28* 863—892

GRIFFIN D R *Listening in the dark* Yale University Press 1958

HUBEL D H and T N WIESEL Receptive fields of single neurons in the cat s striate cortex *J Physiol* (Lond) 1959 *148* 574—591

HUBEL D H and T N WIESEL Receptive fields and functional architecture in the non striate visual areas 18 and 19 of the cat *J Neurophysiol* 1965 *28* 229—289

HUBEL D H and T N WIESEL Receptive fields and functional architecture of monkey striate cortex *J Physiol* (Lond) 1968 *195* 215—243

K[illegible] K

MCCUE J J G Aural pulse compression by bats and humans *J acoust Soc Amer* 1966 *40* 545

MOLLER A R Unit responses in the cochlear nucleus of the rat to pure tones *Acta physiol scand* 1969 a *75* 530—541

MOLLER A R *Unit responses in the cochlear nucleus of the rat to stimulation with pure tones and broad band noise* In preparation 1969 b

NOMOTO M N SUGA and Y KATSUKI Discharge pattern and inhibition of primary auditory nerve fibers in the monkey *J Neurophysiol* 1964 *27* 768—787

NORDMARK J Perception of distance in animal echolocation *Nature* (Lond) 1961 *19*, 1009

SACHS M B and N Y S KIANG Two tone inhibition in auditory nerve fibers *J acoust Soc Amer* 1968 *43* 1120—1128

STROTHER G K Note on the possible use of ultrasonic pulse compression by bats *J acoust Soc Amer* 1961 *33* 696—697

SUGA N Functional properties of auditory neurons in the cortex of echo-locating bats *J Physiol* (Lond) 1965 a *181* 671—700

SUGA N Responses of cortical auditory neurons to frequency modulated sounds in echo-locating bats *Nature* (Lond) 1965 b *206* 890—891

SUGA N Analysis of frequency modulated sounds by auditory neurons of echo-locating bats *J Physiol* (Lond) 1965 c *179* 26—53

VOSS H H Realisierbare Tiefpasse und Bandpasse minimaler Phase mit geebneter Laufzeit und aperiodischem Einschwingverhalten *Frequenz* 1954 *8* 98—102